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低溫下鯉魚肌肉型肌酸激酶之生化及結構研究

Biochemical and Structural studies on the Muscle-Specific Creatine Kinase of the Common Carp (*Cyprinus carpio*) at Low Temperature

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本論文係吳志律君（D91623501）在國立臺灣大學生化科技學系、所完成之博士學位論文，於 2011 年 7 月 25 日承下列考試委員審查通過及口試及格，特此證明

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（是否須簽章依各院系所規定）

誌謝

本研究工作是由吳金洌老師和許祖法老師共同促成的。在研究的過程中個人雖然經歷了許多困難和磨難，但是我認為研究工作的本質當如此，換個新的想法就可讓研究室裡的生活過得更為多采多姿。學習如何面對實驗過程中突如其來地變化，則是另一種值得享受的趣味。然而在這漫長的研究生的日子，充裕經費地提供則必需感謝中研院，國科會，漁業署等各個單位，對於這個研究工作和想法的資助。雖然做為一個博士班研究生，個人的收入十分有限，但是只要秉持樂天知命的態度，應該也沒有機會讓自己陷入困窘的境地。緊張的日子總是會一天天流過，對於曾予我大力協助的同學，學長姊和學弟妹，我實在找不到適當的方法來表達我誠摯的謝意。如果沒有那麼多位曾經和我一起工作的好夥伴，我不知道我是不是可以完成這些有趣的研究成果。不管是工作上或是生活上的互動，一道拼實驗和天馬行空的腦力激盪，都是我研究生活中不可或缺的元素之一，舊方法和老想法的討論常常會衍生出更多新結果和新技術。當然啦，如果沒有我家人自始至終給我的支持，我也沒有辦法完成這個工作。個人不懂得如何表達謝意，然而，謝謝你們。

中文摘要

全球氣候變遷是生物所需面對的一個重要課題。在台灣，每年寒流所造成水產養殖上的經濟損失高達數千萬元以上。魚類生物學家基於魚類逆境生理的分子機制研究，發展出各種技術來降低經濟損失。在此必須先了解硬骨魚類為了應付溫度的變化，發展出避免周遭溫度的傷害的各種生理生化機制。

由於鯉魚可以生存在 35 到 5 °C 之間，之前研究發現鯉魚肌肉型肌酸激酶可以在低溫有較佳的活性，為了了解肌肉型肌酸激酶的分子機制，我們取兩種鯉魚肌肉型肌酸激酶(M1-, M3-CK)和兔子的肌肉型肌酸激酶(RM-CK)來作比較。發現在 15 °C , pH 7.7 以上時，M1-CK 可以比 M3-CK 和 RM-CK 多 3-8 倍的活性。而且，M1-CK 在 pH 8.0 時，在 15 °C 的狀況下具有最高活性，同時 M1-CK 的酵素動力學特性 K_m^{PCr} 和 K_m^{ADP}，在不同溫度和 pH 值下，相對穩定。其催化反應的活化能(E_a)也比較低。從圓二色光譜也發現，M1-CK 在不同的測試溫度和 pH 值下也都維持不變。

當我們將 RM-CK 第 268 個胺基酸，甘胺酸，用 M1-CK 同位置的天冬醯胺酸來取代後，RM-CK G286N 的變異蛋白在 10 °C, pH 8.0 的狀況下，活性為野生型 RM-CK 的 2 倍。動力學特性上，兩者的 K_m 並沒有太大的差異然而圓二色光譜卻發現，RM-CK G286N 在 5 °C, pH 8.0 的狀況下和鯉魚的 M1-CK 極為相似。RM-CK

G286N 結晶的 X 光繞射圖譜解出其受質結合區域的胺基酸互相靠近，反應中心的胱胺酸 283 則從 ADP 的結合部位向外突出。在 pH 7.4-8.0 之間，用體積較小的 dADP 當受質的話，RM-CK G268N 會有較高的反應活性，類似 M1-CK。

接著，我們把 RM-CK 和 M1-CK 的第 268 位置的胺基酸分別突變為天冬胺酸，離胺酸和白胺酸，用以研究這個位置對整個酵素的生物物化特性的影響。其中天冬胺酸和離胺酸變異的肌酸激酶則會有和天冬醯胺酸突變的酵素一樣具有低溫活性，而白胺酸變異的肌酸激酶則沒有。在進行催化反應的緩衝液中加入甘油，可以發現親水性的側鏈有助於穩定酵素低溫下的活性。

綜上所述，我們認為鯉魚 M1-CK 演化出適應低溫環境的功能，從 RM-CK G268N 結構分析發現，其反應中心空間縮小為其具有低溫活性的原因，從動力學和疏水性環境的試驗中得知，第 268 個胺基酸側鏈和水分子的作用會影響酵素的構形，減少該側鏈的疏水性也會同時降低酵素的不穩定性而維持其反應活性。基於這個研究結果，希望可以解開酵素如何在低溫下維持其反應活性的原因。

關鍵字

低溫耐受性; 肌酸激酶; 酵素活性; X 光繞射晶體學; 疏水性。

ABSTRACT

Extreme environmental change is an immediately challenge all over the world. The cold fronts sweeping in the winter, which causes millions of losses in aquaculture, is a severe challenge of Taiwan aquaculture industry. Marine biologists have developed some techniques to minimize the economic loss based on the studies of molecular mechanism of fish in stress. To overcome the change of temperature, physiologically, teleost has developed lots of mechanism to avoid harmful damage of ambient environment. The physiological effects of low temperature have mainly focused on following issues: metabolic compensation, homeoviscous adaptation of biological membranes, and thermal hysteresis.

The common carp could live from 35 to 5 °C. Its muscle-specific creatine kinase (M-CK) could maintain enzymatic activity at temperature around 15 °C. The present studies focus on the three common carp M-CK sub-isoforms (M1-, M2- and M3-CK) which are important in energy homeostasis. Specific activities of the common carp M1-CK were 3 to 8-folds higher than specific activities of M3- and rabbit M-CK at temperatures below 15 °C and pHs above 7.7. K_m^{PCr} and K_m^{ADP} of M1-CK were relatively stable at pHs between 7.1 to 8.0, 25 to 5 °C. Its calculated activation energy of

catalysis (E_a) at pH 8.0 was lower than at pH 7.1. Circular dichroism spectroscopy results showed that changes in secondary structures of M1-CK at the pHs and temperatures under studied were much less than in the cases of rabbit muscle-specific creatine kinase (RM-CK) and M3-CK.

When glycine 268 in RM-CK was substituted with asparagine 268 as found in carp M1-CK, the RM-CK G286N mutant specific activity at pH 8.0, 10 °C was more than 2-fold higher than the wild-type RM-CK at the same condition. Kinetic studies showed that K_m values of the RM-CK G268N mutant were similar to those of the RM-CK, yet circular dichroism spectrum showed that the overall secondary structures of the RM-CK G268N, at pH 8.0, 5 °C, was almost identical to the carp M1-CK enzyme. The X-ray crystal structure of the RM-CK G268N revealed that amino acid residues involved in substrate binding were closer to one another than in the native RM-CK, and the side chain of cysteine 283 in active site of the RM-CK G268N pointed away from the ADP binding site. At pH 7.4-8.0, 35-10 °C, with a smaller substrate, dADP, specific activities of the mutant enzyme were consistently higher than the RM-CK and more similar to the carp M1-CK.

Then, to study the changes in physico-biochemical properties caused by residue 268 in RM-CK and M1-CK at low temperature, six more mutants, aspartic acid 268, lysine 268 or leucine 268 of RM-CK and M1-CK were generated. The peptide

fragments near the active site were found to be phosphorylated. The specific activity results showed that, as in the case of asparagine 268, the aspartic acid 268 and lysine 268 mutants exhibited higher specific activities at low temperature and at higher pH, but not the leucine 268 mutant. The lower hydrophobicity side chain of residue 268 may help the stability of enzyme in glycerol containing buffer.

To sum up, we have found out that, the M1-CK enzyme seems to have evolved to adapt to the synchronized changes in body temperature and intracellular pH of the common carp. The smaller active site of the RM-CK G268N mutant might be one of the reasons for M-CK to improve activity at low temperature. The kinetic results and glycerol influence results indicated that charged side chain of residue 268 of M-CK might cause changes in protein conformation by interacting with water, and decreasing hydrophobicity of M-CK which in turn decreased its instability at low temperature.

KEYWORDS

Cold tolerance; Creatine kinase; Enzyme activity; X-ray crystallography; Hydrophobicity.

ABBREVIATIONS

[E_{total}]:	concentration of total enzyme;
[S]:	substrate concentration;
ADP:	adenosine diphosphate;
AFP:	antifreeze protein;
ATP:	adenosine triphosphate;
B-CK:	brain -specific creatine kinase;
CD:	circular dichroism;
CK:	creatine kinase;
Cr:	creatine;
CSFP:	cold surge frontal passage;
dADP:	2'-deoxyadenosine 5'-diphosphate;
ΔGs:	stabilization free energy;
$\Delta G^{\#}$:	transition state free-energy.
$\Delta H^{\#}$:	transition state enthalpy;
$\Delta S^{\#}$:	transition state entropy;
DTE:	dithioerythreitol
DTT:	1,4-dithio-DL-threitol.

E_a:	Activation energy;
ENSO:	El Nino–Southern Oscillation;
FDA:	Food and Drug Administration;
fMRI:	functional magnetic resonance imaging;
h:	Planck constant;
IAA:	iodoacetamide
k_B:	Boltzmann constant;
k_{cat}:	enzyme reaction rate;
K_m:	Michaelis constant;
K_m^{ADP}:	Michaelis constant of adenosine diphosphate;
K_m^{PCr}:	Michaelis constant of phosphocreatine;
M1-CK (M2-CK, M3-CK):	carp muscle-specific creatine kinase subisoform 1 (2, 3 respectively);
M-CK:	muscle-specific creatine kinase;
MALDI-TOF:	matrix-assisted laser desorption/ionization- time of flight mass spectrometry
MgATP:	adenosine triphosphate, magnesium salt;
PCr:	phosphocreatine;
pK_a:	acid dissociation constant at logarithmic scale;

PMF:	peptide mass fingerprinting
R:	universal gas constant;
RM-CK:	rabbit muscle-specific creatine kinase;
SA:	sinapinic acid
SAS:	solvent accessible surface
SDS-PAGE:	sodium dodecyl sulfate polyacrylamide gel electrophoresis
T:	absolute temperature;
TFA:	triflouroacetic acid
TSAC:	transition-state analog complex;
UV:	ultraviolet;
v:	reaction rate;
V_{max}:	maximum reaction rate;

CONTENT

CHAPTER 1. LITERATURE REVIEW

1.1. GLOBAL CLIMATE CHANGE

Global climate change is a major issue which attracts scientific and public concern. In recent decades, climate has become unstable around the world. Tremendous temperature change might happen with CO_2 accumulation in the atmosphere (Kump, 2009). The stability of biosphere is heavily disrupted by extreme precipitation, drought, warming and cooling (Stuart, 2009). Global warming does not only result in environmental change, but also significant lowering of global temperatures in future have been predicted (Khmelinskii and Stallinga, 2009). Climate change has resulted in numerous shifts in distributions and abundances of species (Pounds, *et al.*, 1999; Parmesan and Yohe, 2003). Animal (Hoffmann and Sgro, 2011; Sanford and Kelly, 2011) and plant (Nicotra, *et al.*, 2010; Ahuja, *et al.*, 2010) need to adapt to extreme environmental variation. Extreme climate resulted in ecological damage makes economic loss in every aspect (Weitzman, 2009). Since the growth of crops could be damaged by extreme temperature and precipitation, the supplement of food could be disrupted (Schlenkera and Roberts, 2009). Biodiversity is vulnerable under environment chaos (Heller and Zavaleta, 2009). Lots of species have become extinct after climate change (Thornton, *et al.*, 2009). Many ecosystems in these areas are losing equilibrium because temperature change promotes desertification in Africa, Latin America and Asia. In Taiwan, a region crossing tropical and sub-tropical areas, extreme climate has occurred more often than usual (Groisman, *et al.*, 2005). There are studies reveal that the mean and variance in the surface average temperature (SAT) in Taiwan have changed significantly since the beginning of the 20th century (Hsu and Chen, 2002). Recently, cold surge frontal passage (CSFP) in

Taiwan occurs more frequently during warm *El Nino*–Southern Oscillation (ENSO) winters. The cold surges in Taiwan have damaged fishery and aquaculture product. In the reports of Fisheries Agency, Council of Agriculture, Taiwan, the aquaculture product loss was NT$ 88,000,000 in Jan 2004, NT$ 140,000,000 in Jan 2005, NT$ 85,000,000 in Feb 2008 and NT$ 63,000,000 in Feb 2011. Some of the most important aquaculture species in Taiwan are not cold tolerance, such as milkfish (*Chanos chanos*), tilapia (*Oreochromis mossambicus*), cobia (*Rachycentron canadum*), grouper (*Epinephelus coioides*) and white shrimp (*Litopenaeus vannamei*).

1.2. FISH THERMO-PHYSIOLOGY UNDER HYPOTHERMIC CONDITION

In order to compensate the stress of low temperature, fish has developed different physiological responses. There are three major strategies in countering low temperature (Hazel, 1993); homeoviscous adaptation in biological membrane (Hazel, 1984), thermal hysteresis (Kristiansen, *et al.*, 2005) and compensation of metabolism (Guderley, 2004). Recently, systematic analyses of cold acclimated fish have been conducted (Ju, *et al.*, 2002; Gracey, *et al.*, 2004).

1.2.1. HOMEOVISCOUS ADAPTATION IN BIOLOGICAL MEMBRANE

Fish, like other poikilotherms, exploit the diversity of lipid structure to fashion membranes with physical properties appropriate to the prevailing ambient temperature. The fluctuation of membrane lipid composition is probably the most ubiquitous and one of the few continuously graded cellular responses to temperature change. To modify lipid composition of subtropical fish to increase cold acclimation ability may be an effective method. The fatty acids compositions in different tissues of fish after adaptation to environmental temperature change have been studied (Wodtke, 1978; Wodtke, 1981; Hazel, 1979; Hazel, 1984; Farkas, *et al.*, 1984).

Essentially, the “homeoviscous adaptation” hypothesis is a widely accepted to explain the temperature-depend physiological adaptation of cell membrane (Macdonald and Cossins, 1985). Membrane fluidity directly affects ions and oxygen transportation, which is critical for survival during temperature change. In the case of carp adaptation to low temperature, changes occurred in the mitochondrial membrane including increase of n-3 and n-6 unsaturated fatty acids contents (Wodtke, 1981) and decrease of α-linolenic acid level

(Wodtke, 1978). In such ways, the fatty acids compositions of diets of fish could contribute to physiological change of cell membrane during environment change (Johnston and Dunn, 1987). Recently, nutritional modification of diets for aquaculture fish is one of the major applications in aquaculture. Changes in lipids addition are reported in regulating fish metabolism (Menoyo, *et al.*, 2004). A tallow-enriched diet has been shown to efficiently increasing the survival period at low temperature (Wu, *et al.*, 2009).

1.2.2. THERMAL HYSTERESIS

Thermal hysteresis is the result of an adsorption of antifreeze proteins (AFP) to the crystal surface (Kristiansen and Zachariassen, 2005). Antifreeze protein of teleost has been found in winter flounder (*Pseudopleuronectes americanus*) in 1989. AFP is a short peptide with poly alanine which interacts to ions and water molecules (Fletcher, *et al.*, 1978). Its conformation is helical and able to bind ice crystal through hydrogen bonding (Houston, *et al.*, 1998).

The AFP gene has been transferred to Atlantic salmon (*Salmo salar*) and produced stable lines of freeze-resistant salmon and other species. On September 19, 2010, Food and Drug Administration (FDA) advisory committee approved the first transgenic farm animal for human consumption, and AquAdvantage Salmon was created by Aqua Bounty Technologies, Inc. After carp M3-CK transgenesis, zebrafish could also maintain its swimming ability at low temperature (Wu, *et al.*, 2011b).

1.2.3. COMPENSATION OF METABOLISM

The common carp (*Cyprinus carpio*) is one of the poikilothermal animal which habitat is

highly variable in temperature from 5° C to more than 35° C (Hwang and Lin, 2002; Stecyk and Farrell, 2006). Since carp is adaptive to a wide range of temperature, physiological responses, especially at low temperature, are interesting in many aspects including metabolism (Wodtke, 1981; Guderley, 1990); neuron response (van der Linden, *et al.*, 2004); endocrine release (Flik, *et al.*, 2006) and behavior (Rome, *et al.*, 1990; Johnston and Temple, 2002). Studies on carp cold acclimation responses have revealed the compensatory responses of activities of glycolytic and tricarboxylic acid cycle enzymes in muscle (Guderley, 1990). The increased polyunsaturation of mitochondrial membranes should raise rates of mitochondrial respiration for fish living at low temperature (Guderley, 2004). Functional magnetic resonance imaging (fMRI) study on the brain of common carp reveals that the primary sensory trigeminal nucleus, the valvula cerebelli and some motornuclei, is activated during temperature drop (van den Burg, *et al.*, 2006). In some of these studies, the energy supplement is the most important criterion to maintain fish living at low temperature.

In the common carp, blood and intracellular pH rises from 7.6 to 8.0 when water temperature decreases from 20 to 10 °C, and it could change 0.4 pH units within a day (Reeves, 1977; Rothe and Heisler, 1979; Albers, *et al.*, 1983; Moyes, *et al.*, 1988). It has been known that the pH of extra- and intracellular fluids of poikilothermal animals varies inversely with body temperature (Yancey and Somero, 1978). At this broad range of temperature, energy supplement is an important criterion to maintain live of the common carp.

1.3. CREATINE KINASE

Creatine kinase (CK, EC 2.7.3.2) is one of the most important enzymes involved in energy homeostasis, and there are four different isozymes in this enzyme family. It belongs to phosphagen kinases family. Muscle-specific creatine kinase (M-CK) catalyzes the reversible reaction that converts phosphocreatine (PCr) and ADP to creatine (Cr) and ATP and thus provides ATP for muscle contraction (Wyss, *et al.*, 1991; McLeish and Kenyon, 2005). The M-CK is specific for differentiated muscle, whereas the brain form CK (B-CK) is found in brain and a variety of other tissues, and these cytosolic forms are used mostly in providing ATP for muscle contraction (Schlegel, *et al.*, 1988; Qin, *et al.*, 1998).

1.3.1. ENZYME CHARACTERISTICS

CKs are highly conserved in vertebrates. The M-CK of homeothermal rabbit (*Oryctolagus cuniculus*) (RM-CK) has been cloned and its crystal structure has been resolved (Putney, *et al.*, 1984; Rao, *et al.*, 1998). The specific activity of RM-CK is highest at around 35 °C to 40 °C, and then decreases as temperature decreases.

Previously, three carp M-CK subisoforms, M1-, M2-, and M3-CK, have been cloned (Sun, *et al.*, 1998). Immunoblot and immunohistochemical analyses show that all three M-CKs were detected in red and white muscle of different temperature (30-10 °C) acclimatized carp (Sun, *et al.*, 2002). It would be interesting to elucidate the possible physiological functions of the three carp subisoforms by studying the temperature and pH dependent changes of their biochemical properties.

Since M-CK is closely related to muscle function, assay of CK has become an important clinical diagnosis. Assay of CK for disease diagnoses include cardiac diseases (Wu, 2005),

muscular dystrophy (Hsu, 2004), and inflammatory responses (Nirmalananthan, 2004). Biochemical analysis of serum CK activity has been developed since 1965, and most of these methods involve the assay of coupled enzymes or measurement of proton formation (Holliday, *et al.*, 1965). However, under variable temperature and pH conditions, these methods would not be suitable to determinate the specific activities of the subisoforms of carp M-CK (Tanzer and Gilvarg, 1959; Mahowald, *et al.*, 1962; Rosalki, *et al.*, 1967).

1.3.2. ENZYME STRUCTURE

The primary structures of RM-CK and carp M1-CK share 86% identity (Figure 1). Circular dichroism (CD) spectroscopy has been employed to study the impact of intra-subunit domain-domain interactions on RM-CK (Zhao, *et al.*, 2006a; Zhao, *et al.*, 2006b). Well conserved residues have been studied by site directed mutagenesis and chemical modification (Chen, *et al.*, 1996). The cysteine 283 residue is important in keeping creatine anchored and positioned for nucleophilic attack on the γ-phosphorus of MgATP within a SN2 type reaction (Milner-White and Watts, 1971; Lahiri, *et al.*, 2002) (Figure 2a). Arginine and histidine are thought to be important in stabilizing ADP/ATP as well as involved in acid/base catalyst generation (Cantwell, *et al.*, 2001). Arginines 96, 132, 236, 292 and 341 are correlated to nucleotide binding, and valine 72, glutamic acid 232, glutamic acid 241 and aspartic acid 326 are correlated to creatine binding which have been reported to be transition-state analog complex (TSAC) binding residues, and histidine 296 participates in catalysis (Chen, *et al.*, 1996; Forstner, *et al.*, 1997; Wood, *et al.*, 1998; Edmiston, *et al.*, 2001; Jourden, *et al.*, 2005; Uda, *et al.*, 2009). Two flexible loops, residues 60-65 and 316-326, control the entrance of creatine or phosphocreatine, and creatine interacts with valine 72, aspartic acid 232 and aspartic acid 326, and cysteine 283 is important but not essential for catalysis (Forstner, *et al.*,

1996; Cantwell, *et al.*, 2001; Reddy, *et al.*, 2003; Tanaka and Suzuki, 2004). Crystal structures of human M-CK and RM-CK have been determined (Rao, *et al.*, 1998; Shen, *et al.*, 2001) (Figure 2b, c). The high-resolution RM-CK structure shows that aspartic acid 55 in the RM-CK-ADP subunit forms a hydrogen bond to the side chain of histidine 7 in the adjacent RM-CK-TSAC subunit, while aspartic acid 55 from the RM-CK-TSAC subunit forms a hydrogen bond to the backbone N atom of proline 2 within the same chain (Ohren, *et al.*, 2007). It has been proposed that the loss of this hydrogen bond was sufficient to cause dissociation of the dimer. In the case of M3-CK, both the proline 2 and histidine 7 are absent in the N-terminal, thus, it might be the reason for the thermal instability of this enzyme (Sun, *et al.*, 1998).

The CKs of different species have been studied for employing site directed mutagenesis studies (Gross, *et al.*, 1994; Khuchua, *et al.*, 1998; Perraut, *et al.*, 1998; Cox, *et al.*, 2003; Guo, *et al.*, 2003). The mutant sites located in substrate binding sites have also been studied (Eder, *et al.*, 2000; Mourad-Terzian, *et al.*, 2000; Edmiston, *et al.*, 2001; Uda, *et al.*, 2004; Ohren, *et al.*, 2007). Most of the mutants generated shows decrease in activity of CK.

In M-CK, cysteine 283 forms part of a conserved cysteine-proline-serine motif and has a pK_a about 3 pH units below that of a regular cysteine residue. Results of computational and UV difference spectroscopy studies of wild-type human M-CK and its P284A, S285A and C283S/S285C mutants, show that serine 285 lowers the pK_a about 1 pH unit and proline 284 lowers it a further 1 pH unit (Cook, *et al.*, 1981; Wang, *et al.*, 2006). Takes together, change in ambient temperature affects carp body temperature and intracellular pH which in turn affects the pK_a of carp M1-CK (Albers, *et al.*, 1983).

1.3.3. THERMODYNAMIC

Phosphagen kinases exhibit different biochemical characteristics under different temperature. K_m^{ATP} of arginine kinase of *Homarus vulgaris* has been reported to show no difference between 20 to -25 °C, while using a mitochondrial respiration assay, K_m^{ADP} of tortoise CK decreased at low temperature (Travers, *et al.*, 1978; Birkedal and Gesser, 2004). Recently, the M-CK of icefish (*Chaenocephalus aceratu*) has been found to function best at 0.5 °C (Winnard, *et al.*, 2003). Biochemical kinetic properties, crystallographic analysis, and cryoenzymology of phosphagen kinases have been studied extensively, yet, how CK functions in order for eurythermic fishes to adapt to different ambient temperature remains to be elucidated (Travers and Barman, 1995; McLeish and Kenyon, 2005).

1.3.4. ENZYME STABILITY

Enzyme activity decreases when deviates from optimum temperature. Psychrophilic enzyme has been suggested to obtaining higher flexibility structure than thermophilic or mesophilic enzyme (Feller and Gerday, 2003). Most psychrophilic enzymes' active sites are more flexible, resulting in lower K_m, and low-activation enthalpy. They are also more active and stable at lower temperature (Siddiqui and Cavicchioli, 2006). The cold adapted enzyme activity may illustrated by the theoretical and practical thermodynamic parameters (Lonhienne, *et al.*, 2000). Low temperature results in cold denaturation of proteins. Cold denaturation has been attributed to an increase in the solubility of the hydrophobic residues in aqueous solutions at low temperatures (Jaenicke, 1990; Privalov, 1990). A hydrophilic side chain may reduce the hydrophobicity on the interface of protein and solvent (Timasheff, 1993; Folch, *et al.*, 2010). In previous studies, folding of CK is obviously influenced by salt, organic

solvent and denaturants (Saks, *et al.*, 1986; Yang, *et al.*, 1997; Huang, *et al.*, 2001; Du, *et al.*, 2006). Glycerol is one of the substances which reduced the RM-CK activity (Feng and Yan, 2008). It also reduces the formation of hydrogen bond between water molecules (Bhatnagar, *et al.*, 2007). In this way, glycerol helps protein stability at low temperature by preventing ice-crystal formation and water sphere around protein (Wang, *et al.*, 2008).

Since viscosity of reaction solution is an important factor to influence enzyme activity, glycerol is applied to modify solution viscosity (Barbier and Campbell, 2005). Glycerol may reduce hydrogen bond formation between protein and solution.

The hydrogen bond formation between water molecules is supposed to reduce entropy of solution (Meng, *et al.*, 2004). Glycerol may reduce the stabilization free energy (ΔGs) for less than 0.9 kcal/mole protein. However, at high concentration, glycerol may reduce enzyme activity. Considering the solution environment interaction, hydrogen bond formation between enzyme and H_2O is compatible to those between two H_2O molecules. Without binding water at low temperature, enzyme will become unstable. Water bulk formation with hydrogen bonds network may reduce free energy of unstable enzyme (Tsai, *et al.*, 2002). Adding cosolvent, such as glycerol, may help to stable the enzyme by keeping the enzyme surface H-bond and *ven der waal's* force, interrupt hydrogen bond formation of H_2O molecules and prevent the hydrophobic force of enzyme itself (Vagenende, *et al.*, 2009). Low temperature results in cold denaturation of proteins.

1.4. AIM OF STUDY

FIND OUT THE REGULATION MECHANISM OF MUSCLE-SPECIFIC CREATINE KINASE ACTIVITY AT LOW TEMPERATURE.

There are three topics mentioned.

1. Study the differences in activity of M-CKs at low temperature.

2. Identify the possible structural differences correlated to the M-CKs activity at low temperature.

3. Study M-CK structures and activities differences at low temperature.

CHAPTER 2. MATERIAL AND METHODS

2.1. CARP M-CKS ACTIVITY DETERMINATION

2.1.1. SAMPLE PREPARATION

Carp M1-, M2-, and M3-CK cDNAs were constructed as described previously (Sun, *et al.*, 1998). M1-, M2-, and M3-CK recombinant plasmids (pET28a) were transformed to *E. coli* BL21(DE3)pLysS and cultured in 1L LB medium (pH 7.6). After induction, the culture medium was harvested and pellet resuspended in binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). After sonication and centrifugation at 12,000 xg, 15 minutes, supernatant was filtrated using a 0.45 μm filter and applied into a nickel column. The recombinant protein was eluted with elution buffer (0.2 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). Eluate was dialysed in 30 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM β-mercaptoethanol, pH 7.1 at 4 °C for 1 day, and concentrated with an Amicon Ultra 15 filtration tube (10,000 mwco, Millipore). The concentrated protein solution was stored at -30 °C with glycerol (protein solution : glycerol, v/v, 4:1). Protein concentration was estimated using the Commassie plus^{TM} protein Assay Reagent (BioRad). RM-CK was a commercial product (Merck, Ltd. Co.), and this enzyme was dissolved in 30 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM β-mercaptoethanol, pH 7.1 and stored at -30 °C with glycerol (protein solution : glycerol, v/v, 4:1).

2.1.2. SPECIFIC ACTIVITY AND THERMAL INACTIVATION ASSAY

M-CK activity assay was based on the method by Hughes and modified to fit different assay conditions (Hughes, 1962). Since the Sigma M-CK activity assay kit (SIGMA Diagnostic, No. 520), which was used in our previous studies, was no longer available, $MgCl_2$, HCl, ADP (potassium salt), and phosphocreatine (PCr, disodium salt) were purchased from Merck; p-hydroxyl-mercuribenzoate, α-naphthol, and diacetyl were from Sigma; Tris was from Boehringer; (1, 4)-dithiothreitol (DTT) was from Amresco. All chemicals were of analytical or biotech grade and all the stock solutions were prepared as mentioned in the Sigma assay kit. The basal reaction buffer of M-CK activity assay was 30 mM Tris-HCl, 10 mM $MgCl_2$, and 1 mM DTT. pHs of the assay buffers were adjusted with 1 M HCl at the assay temperatures. M-CK activity assays were carried out at pH 7.1, 7.4, 7.7, or 8.0 and at temperatures 35, 30, 25, 20, 15, 10, 5, or 0 °C. Assay solution also contained 12.5 mM PCr, 1 mM ADP, and enzyme concentrations in the range of 0.0125-0.05ng in 80 μl reaction volume. After 15 minutes of enzyme reaction, *p*-hydroxyl-mercuribenzoate (50 mM, 20 μl) was added to terminate the reaction, then α-naphthol (2%, w/v; 100 μl) and diacetyl (1:200, v/v; 100 μl) were added for the colorimetric reaction and spectrophotometric absorption was measured at 520 nm. Creatine produced in each reaction was quantified using a creatine standard curve. Enzyme specific activity U is defined as 1 μmole of creatine formed per minute per milligram of enzyme.

Thermal stability experiments were carried out in the activity assay buffer at pH 7.1 or 8.0 with enzyme concentration at 0.0025 $\mu g \cdot \mu l^{-1}$. The assay reaction was preincubated in the absence of ADP and PCr at 10, 20, 30, 40, 50, 60, or 70 °C for 30 minutes, then, cooled on ice before ADP and PCr were added and assayed at 30 °C for 15 minutes. At least 3 repeats were carried out, and the highest specific activity was taken as 100% (Zhao, *et al.*, 2006a). The specific activities of all the M-CKs in each condition were assayed from three different

batches of recombinant protein preparation and each preparation assayed for more than three times.

2.1.3. KINETIC ASSAY AND DATA ANALYSIS

Kinetic analyses of M-CKs were carried out as described by Cleland (Cleland, 1979). For biochemical kinetic analyses, the same specific activity assay mentioned above was carried out at pH 7.1 or 8.0, and at 25, 15 or 5 °C. ADP concentrations ranged from 0.1 to 1 mM with PCr concentration at 12.5 mM for RM-CK, and 100 mM for M1- and M3-CKs when determining K_m^{ADP}. PCr concentrations ranged from 0.5 to 100 mM with ADP concentration at 1 mM when determining K_m^{PCr}, and k_{cat}, K_m^{ADP} and K_m^{PCr} were calculated using the Michaelis-Menten equation (Marangoni, 2003). K_m and k_{cat} were obtained using these formulae. Reaction time was 7 minutes for the 25 °C, 15 minutes for the 15 °C, and 45 minutes for the 5 °C reactions. Reaction rates were determined as description in the previous paragraph. All the results were repeated with different batches of recombinant protein. The molecular weights were calculated using the free website ExPASy Proteomics Server, and concentrations of the enzymes were calculated using these data.

(d ln k_{cat} / dT) can be derived from the kinetic analysis of CK by the Michaelis-Menten equation (Marangoni, 2003)

$$v = V_{max}\,[S] \cdot (K_m + [S])^{-1} \qquad \text{(eq. 1)}$$

where v is reaction rate obtained from Cr formation rate, [S] is the initial concentration of one of the substrates while the other substrate concentration is fixed**,** V_{max} is defined as the maximum reaction velocity,

$$V_{max} = k_{cat}\,[E_{total}] \qquad \text{(eq. 2)}$$

In the steady-state case,

$$v^{-1} = K_m \cdot [S]^{-1} \cdot k_{cat}^{-1} \cdot [E_{total}]^{-1} + k_{cat}^{-1} \cdot [E_{total}]^{-1} \quad \text{(eq. 3)}$$

and when the graph v^{-1} vs. $[S]^{-1}$ is plotted, when $[S]^{-1} = 0$, $v^{-1} = k_{cat}^{-1} \cdot [E_{total}]^{-1}$, and when $v^{-1} = 0$, $[S]^{-1} = - K_m^{-1}$.

2.1.4. CIRCULAR DICHROISM SPECTROSCOPY

Circular dichroism spectra were recorded on a Jasco J715 spectropolarimeter at temperatures 5, 10, 15, 20, 25, 30, 35, 40, 45, or 50 °C (Kelly, *et al.*, 2005). For far-UV spectrum, 1 mm path length quartz cuvette of 200 μl was used at a scan speed of 20 nm · min^{-1} from 200 nm to 250 nm. The CD solution contained 2.32 μM of M-CK protein in the activity assay buffer at pH 7.1 or 8.0 in the absence of ADP and PCr. Data were collected per 0.1 nm and an average of 10 spectra was corrected by subtraction of spectra recorded on the activity assay buffer in the absence of enzyme. Raw data were smoothed by moving average of 19 nearby data.

2.1.5. CALCULATION OF ACTIVE ENERGY (*Ea*)

A cold-adaptive enzyme exhibits high activity at low temperature by decreasing activation free-energy ($\Delta G^{\#}$) barrier between ground state (substrate) and transition state ($T\Delta S^{\#}$) (Siddiqui and Cavicchioli, 2006).

From the Arrhenius equation,

$$k_{cat} = A\, e^{-Ea / RT} \quad \text{(eq. 4)}$$

where k_{cat} is the enzyme reaction rate, T is absolute temperature, *Ea* is activation energy,

A is the preexponential factor and R is the universal gas constant (8.314 J mol^{-1} K^{-1}). After natural logarithmic in each side of equation 4,

$$\ln k_{cat} = - Ea / RT \quad \text{(eq.5)}$$

Then, equation 5 is differential by temperature (T),

$$d \ln k_{cat} / dT = Ea / RT^2 \quad \text{(eq. 6)}$$

On the other hand, by the "Transition state theory",

$$k_{cat} = (k_B T/h)\, e^{-\Delta G^{\#} / RT} \quad \text{(eq. 7)}$$

$$\Delta G^{\#} = \Delta H^{\#} - T\Delta S^{\#} \quad \text{(eq. 8)}$$

Where $\Delta G^{\#}$ is transition state free energy, $\Delta H^{\#}$ is change in activation enthalpy, $\Delta S^{\#}$ is change in activation entropy and T is absolute temperature. k_B is Boltzman constant (1.38×10^{-23} J K^{-1}) and *h* is Planck constant (6.63×10^{-34} J s).

Replace $\Delta G^{\#}$ by $\Delta H^{\#} - T\Delta S^{\#}$, equation 7 will become

$$\ln k_{cat} = \ln (k_B T / h) - (\Delta H^{\#} / RT) + (\Delta S^{\#} / R) \quad \text{(eq. 9)}$$

Then, equation 9 is differential by temperature (T),

$$d \ln k_{cat} / dT = (1 / T) + (\Delta H^{\#} / RT^2) = (\Delta H^{\#} + RT) / (RT^2) \quad \text{(eq. 10)}$$

Combine equation 6 and equation 10,

$$\Delta H^{\#} = Ea - RT \quad \text{(eq. 11)}$$

In this way, $\Delta G^{\#}$, $\Delta H^{\#}$, $\Delta S^{\#}$ and *Ea* can be calculated. The thermodynamic studies of cold adapted enzymes are one of the most attractive parts to analysis the function and structure correlation (D'Amico, *et al.*, 2002).

2.2. COMPARISON OF RM-CK AND M1-CK

2.2.1. CONSTRUCTION AND PRODUCTION OF RM-CK MUTANTS

A rabbit muscle cDNA library was purchased from Merck, and RM-CK was cloned with primer pairs, 5'-ATCCCATATGCCGTTCGGCAAC-3' and 5'-AAAACTCGAGCTACTTCTGGGC-3'. The PCR product was digested with *NdeI* and *XhoI*, and then ligated into pET28a. Bridge PCR method was used in site directed mutagenesis on RM-CK-pET28a clone with internal primer pairs for mutation sites, 5'-TGAAGGGGTGCCCATGTTTCTT-3' and 5'-AGATCTTTAAGAAACATGGGCA-3' for RM-CK A267H; 5'-CACATGAAGGGGTGGTTAGCTTTC-3' and 5'-CTTTAAGAAAGCTAACCACCCC-3' for RM-CK G268N; 5'-TCATTCCACATGAAGCCGTGCC-3' and 5'-AAAGCTGGGCACGGCTTCAT-3' for RM-CK P270G; 5'-CGGGTGAGAATCTCGTCGAACTTGGGG-3' and 5'-CCCCAAGTTCGACGAGATTCTCACCCG-3' for RM-CK A329S. The constructs were transformed into *E. coli* BL21 (DE3) pLysS and cultured for protein production as described previously. M1-CK was constructed as described previously, and M1-CK N268G was also constructed by bridge PCR with internal primer pairs, 5'-TTCAAGAAGCACGGCCATGGATTCATG-3' and 5'-CATGAATCCATGGCCGTGCTTCTTGAA-3' (Meza, *et al.*, 1996). All the constructs were digested with *NdeI* and *XhoI* and ligated into pET28a. All the clones were verified by sequencing. All the protein products were stocked with 50% glycerol at -30 °C. Concentration of protein was determined by Commassie Plus protein assay reagent (BioRad).

Recombinant enzymes were produced as described in section 2.1.1.

2.2.2. SPECIFIC ACTIVITY AND THERMAL INACTIVATION ASSAY

M-CK specific activity assay was based on the method described by Hughes and modified to fit different assay conditions as described in section 2.1.2.

2.2.3. KINETIC ASSAY AND DATA ANALYSIS

Kinetic analyses of CKs were carried out as described in Cleland, 1979 as described in section 2.1.3.

2.2.4 CIRCULAR DICHROISM SPECTROSCOPY

CD spectra were recorded on a Jasco J715 spectropolarimeter at temperatures 35 °C, 25 °C, 15 °C or 5 °C, as described in 2.1.4.

2.2.5. CRYSTALLIZATION AND X-RAY DIFFRACTION DATA COLLECTION

The hanging-drop vapor-diffusion method at 25 °C was used for initial crystallization screening of RM-CK G268N and M1-CK. RM-CK G268N crystals grew reproducibly with 10 mg/ml protein in storage solution (30 mM Tris-base, 10 mM $MgCl_2$, 1 mM DTT, pH 7.1, 1 µl) and reservoir solution (20% PEG 3350, 190 mM tripotassium citrate, 0.5 µl), and extended using a Silver Bullets Bio kit (Hampton Research) (0.16% Glycyl-glycyl-glycyl-glycine, 0.16% L-Leucyl-glycyl-glycine, 0.16% Met-Ala-Ser, 0.16% Glycyl-glycyl-glycyl-glycyl-glycine, 0.16% Ala-Leu, 0.16% Tyr-Leu, 20 mM HEPES, pH 6.8,

0.5 μl) (Yeh, *et al.*, 2007). The RM-CK G268N crystals grew as hexagonal pillars with dimensions of 0.3 mm × 0.3 mm × 0.8 mm. The crystals were cryoprotected in liquid nitrogen by quick transfer from the hanging drop before data collection. All data sets were taken on a Rigaku R-AXIS IV++ imaging plate detector system using double-mirror-focused CuKα X-ray radiation generated from a Rigaku RU-300 rotating anode operating at 50 kV and 80 mA. Data were indexed, integrated, and scaled using HKL 2000 software packages (Liu, *et al.*, 2004).

2.2.6. STRUCTURE DETERMINATION AND REFINEMENT

The initial phases of RM-CK G268N were solved by using the molecular replacement software MOLREP of the Collaborative Computational Project, Number 4 (CCP4) suite using a truncated PDB (1crk) as search model (CCP4, 1994). After initial model refinements by REFMACS (Murshudov, *et al.*, 1997), the final model for the RM-CK G268N consisted of 381 amino acid residues from methionine 1 to lysine 381. The R-factor of this model for all reflections was between 127 and 3.3 Å. The R factor of this structure was refined to 19.9%, and the R_{free} value of 26% was obtained by using 5.0% randomly distributed reflections. The Ramachandran plots for both structures did not violate accepted backbone torsion angles. The PDBviewer 4.01 program was used to generate figures.

2.2.7. SUBSTRATE SUBSTITUTION

2'-deoxyadenosine-5'-diphosphate (dADP, 32 mM) and glutathione (reduced form, 8 mM) were dissolved in distilled water as stock solution (Lee, *et al.*, 1977). Assay solutions for CK specific activity and kinetic analysis with dADP were as described above. There were at least

3 repeats in each assay condition and with more than 1 batch of recombinant enzyme products.

2.2.8. CALCULATION OF *pKa*

Using data processing methods described in Cleland (Cleland, 1979), the pH profiles of active site that showed a decrease in log V_{max}/K_m as pHs decrease are fitted to equation 12,

$$\log (V_{max}/K_m) = \log [C / (1 + H / K_1)] \quad \text{(eq. 12)}$$

while the pH profiles that showed a decrease in log V_{max}/K_m as pHs increase are fitted to equation 13,

$$\log (V_{max}/K_m) = \log [C / (1 + K_1 / H)] \quad \text{(eq. 13)}$$

C is the pH independent value of V_{max}/K_m, H is the concentration of hydrogen ions and K_1 represents the dissociation constant of a specific group of the enzyme (Wang *et al.*, 2006). In this study the resulting K_1 values are the apparent pK_a of the thiol group of cysteine 283 of RM-CK, M1-CK and their mutants at different temperatures.

2.2.9. STATISTICAL ANALYSIS

Statistical analysis of specific activity and kinetic results are presented as mean ± standard deviation. One-way ANOVA ($P < 0.05$) method was used in analysis of differences between assays.

2.3. IDENTIFY THE POSSIBLE ROLE OF RESIDUE 268 OF M-CK

2.3.1. CONSTRUCTION OF SITE-DIRECTED MUTAGENESIS OF RM-CK AND M1-CK MUTANTS

A rabbit muscle cDNA library was purchased from Merck, and RM-CK was cloned with primer pairs, 5′-ATCCCATATGCCGTTCGGCAAC-3′ and 5′-AAAACTCGAGCTACTTCTGGGC-3′. The PCR product was digested with *NdeI* and *XhoI*, and then ligated into pET28a (Novagen). M1-CK was construction previously; it is also digested with *NdeI* and *XhoI* (Fermentas), and then ligated into pET28a.

To mutated glycine to asparagines, aspartic acid, lysine or leucine at residue 268 of RM-CK and M1-CK, bridge PCR method was used in site directed mutagenesis. The primers were displayed in Table 1.

The cloning procedures were described in section 2.2.1.

2.3.2. EXPRESSION AND PURIFICATION OF RM-CK, M1-CK AND MUTANTS PROTEINS

The protein producing procedures were described in section 2.1.1.

2.3.3. MASS SPECTROMETRY ANALYSIS

Protein sample were treated with in-gel digestion before mass spectrometry analysis. Protein sample was loaded to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Following electrophoresis, the gel stained with coomassie brilliant blue G-250

(MERCK). Excise protein spot from SDS-PAGE, transfer the gel slices into a 1.5 ml microcentrifuge tube. Add 100 μl of 50 mM dithioerythreitol (DTE) / 25 mM ammonium bicarbonate (pH 8.5) to the gel sample for reduction, and then soak for 1 hour at 37 °C. Centrifuge at 10,000 xg for 1 minute, remove DTE completely. Add 100 μl of 100 mM iodoacetamide (IAA) / 25 mM ammonium bicarbonate (pH 8.5) to the gel sample for alkylation, and then soak for 1 hour at room temperature at dark. Centrifuge at 10,000xg for 1 minute, remove IAA completely. Add 100 μl of 50% acetonitrile / 25 mM ammonium bicarbonate (pH 8.5), soak for 15 minutes. Centrifuge at 10,000 xg for 1 minute, remove buffer completely. Soak the gel in 100 μl of 100% acetonitrile for 5 minutes and centrifuge at 10,000 xg for 1 minutes, remove acetonitrile. Dry the gel slice for 5 minute in oven. Add 0.1 μg trypsin / 10 μl 25 mM ammonium bicarbonate (pH 8.5), spin down the gel pieces/trypsin solution and incubate at 37 °C for at least 16 hours. Add 50 μl acetonitrile / 5% triflouroacetic acid (TFA) to gel sample. Sonicate the sample for 10 seconds, and then stop for 10 seconds. This process repeat 10 times. Spin down the gel pieces at 10,000 xg for 1 minute. Aspirate the supernatant from the sample tube and transfer to the new tubes. Finally, concentrate in a speed vac (Thermo Savant SC210A Speed Vac Plus) at 30 °C to 1-2 μl.

Mix 0.5 μl analyte solution with 0.5 μl sinapinic acid (SA) solution; apply the 0.5 μl mixture on standard steel target (MTP 384 target plate ground steel T F, Bruker) and drying at room temperature. The standard (Bruker, NO. 206355) is also applied 0.5 μl on standard steel target. Microfle LRF, MALDI-TOF (Bruker) was used for peptide mass fingerprinting (PMF) for protein identification. The system was operated under BioToolsTM and raw MS data were processed for database searching using Mascot Search. (Yeh, *et al.*, 2006)

2.3.4. CIRCULAR DICHROISM

CD spectra were recorded on a Jasco J715 spectropolarimeter (Jasco International, Tokyo, Japan) at temperatures 35 °C and 15 °C, as described in 2.1.4.

2.3.5. SPECIFIC ACTIVITY ASSAY

M-CK specific activity assay was based on the method described by Hughes and modified to fit different assay conditions as described in section 2.3.2.

The assay of different glycerol concentration was preceded as previous method. In order to manipulate glycerol, 5 μl, 200 mM PCr and 5 μl, 16 mM ADP were used. 20 μl of different concentrations (glycerol/H_2O, w/w; 0%, 5%, 10%, 20%, 30%, 40%, 60%, 80%) of glycerol were added in the reaction buffers of pH 7.1 and 8.0. Specific activity U was defined as 1 μmole of creatine formed per min per mg of enzyme. All the reactions were repeated at least 3 times and with more than 1 batch of recombinant proteins.

2.3.6. KINETIC ASSAY AND DATA ANALYSIS

Kinetic analyses of CKs were carried out as described in 2.1.3.

2.5.7. HYDROPHOBICITY INDEX

Hydrophobicity indexes of different M-CKs and mutants were calculated based on the Kyte and Doolittle method (Kyte and Doolittle, 1982). An online hydrophobicity index analysis program was offered by Marc Offman in "http://bmm.cancerresearchuk.org/~offman01/ hydro.html". The window length is 6.

2.3.8. PROTEIN MODELING

RM-CK G268N structure determination and refinement was described previously (Wu, *et al.*, 2011a). RM-CK structure (2crk)(Rao, *et al.*, 1998) was approached from PDB database. PyMOL 1.1 was performed to raise protein modeling. The solvent accessible surface area was calculated with PyMOL's program.

2.3.9 STATISTICAL ANALYSIS

Statistical analysis of specific activity and kinetic results were described in section 2.2.8.

CHAPTER 3. RESULTS

3.1. THE CARP M1 MUSCLE-SPECIFIC CREATINE KINASE SUBISOFORM IS ADAPTIVE TO THE SYNCHRONIZED CHANGES IN BODY TEMPERATURE AND INTRACELLULAR PH THAT OCCUR IN THE COMMON CARP

3.1.1. M1-CK WAS MORE ACTIVE AT AND BELOW 30 °C THAN RM-, M2-, AND M3-CK

The assay condition at pH 7.1 that allowed M1-CK to exhibit highest specific activity at 30 °C was taken as the basal condition. Sodium chloride and potassium chloride reduced M1-CK activity even at 5 mM. RM-, M1-, M2-, and M3-CK activities were assayed at 4 different pHs and 8 different temperatures. RM-CK showed its highest specific activity of 327.5 ± 8.2 U at pH 7.1, 35 °C (Figure 3a). Its activity started to decrease at temperatures above and below 35 °C. M1- and M3-CK showed their highest activities of 287.1 ± 15.0 and 142.6 ± 16.6 U, respectively, at pH 7.1, 30 °C. However, M2-CK specific activities were below 40 U at all pHs and temperatures tested, therefore, its biochemical characteristics were not pursued further. At pH 7.1, 15 °C, RM- and M3-CK lost more than 90% of their enzymatic activities, while M1-CK lost 73%. However, M1-CK seemed to be able to maintain its activity between 15 to 10 °C before it suffered a further loss at 5 °C.

At pH 7.4, 30 °C, activities of all three enzymes decreased slightly (Figure 3b). Activity of M3-CK did not change between 25 to 15 °C, while activity of M1-CK also showed a plateau between 20 to 10 °C. At pH 7.7, M1-CK activity remained stable from 30 to 15 °C, while activity of M3-CK increased from 30 to 20 °C then decreased when below 20 °C

(Figure 3c). At pH 8.0, activities of RM- and M3-CK were significantly lower, yet M1-CK activity remained stable from 30 to 20 °C, and then increased significantly at 15 °C (Figure 3d). M1-CK was able to maintain its activity around 150 U at 15 °C, and even at 0 °C, its activity of almost 50 U was 60% the M-CK activity of icefish at 0 °C (Winnard, *et al.*, 2003). Therefore, it seemed that M1-CK can function well in carp muscle from 35 to 5 °C.

3.1.2. K_m^{PCr} AND K_m^{ADP} OF M1-CK CHANGED LITTLE AT DIFFERENT TEMPERATURES

Since the differences in activities between RM-, M1-, and M3-CK were most significant at pHs 7.1 and 8.0, and M1-CK showed interesting activity feature at pH 8.0, 15 °C, determination of K_m^{PCr} and K_m^{ADP} was carried out at these two pHs and at temperatures 25, 15, and 5 °C. At pH 7.1, 25 °C, K_m^{PCr} of RM-, M1-, and M3-CK were 1.25 ± 0.02, 6.32 ± 0.72, and 7.70 ± 0.51 mM, respectively (Figure 4a). K_m^{PCr} of RM-, and M1-CK increased when temperature decreased from 25 to 5 °C. In the case of M3-CK, K_m^{PCr} from 25 to 15 °C was at similar range to the other two enzymes then climbed steeply from 15.17 ± 0.99 mM at 15 °C to 76.91 ± 3.88 mM at 5 °C. This implied a significant conformational change in the protein structure of the M3-CK. At pH 8.0, when temperature decreased from 25 to 5 °C, K_m^{PCr} of RM-CK and M3-CK decreased slightly, while the M1-CK values seemed unchanged (Figure 4b). At pH 7.1, K_m^{ADP} of RM-CK decreased steeply from 25 to 15 °C then leveled off from 15 to 5 °C, and M3-CK increased from 25 to 15 °C then leveled off from 15 to 5 °C (Figure 4c). At this pH, the values of K_m^{ADP} of M1-CK changed little throughout. At pH 8.0, from 25 to 5 °C, the changes in K_m^{ADP} of M3-CK were similar to those at pH 7.1, while K_m^{ADP} of RM- and M1-CK varied little throughout (Figure 4d).

3.1.3. SPECIFICITY CONSTANT (k_{cat}/K_m) AND ACTIVATION ENERGY OF CATALYSIS (*Ea*) OF RM-, M1-, AND M3-CK

Due to high K_m^{PCr} of the three enzymes at low temperature, it was impossible to carry out specific activity assay for k_{cat} calculation at 5x K_m^{PCr}. Therefore, the highest concentration of PCr was set at 100 mM, which was higher than intracellular concentration of PCr (Iyengar, *et al.*, 1985). k_{cat} calculation showed that at both pHs 7.1 and 8.0, from 25 to 5 °C, the k_{cat} values of RM-CK were more affected by decrease in temperature, while at pH 8.0, the k_{cat} values of M1-CK decreased least (Table 2).

Catalytic efficiency (k_{cat}/K_m^{PCr}) of the three enzymes decreased when temperature decreased (Table 2). And, k_{cat}/K_m^{PCr} of M1-CK did not show any advantage over RM- and M3-CK, except at 5 °C. At pH 7.1, between 25 to 15 °C, k_{cat}/K_m^{ADP} showed that M3-CK seemed to be the most efficient enzyme among the three enzymes. While curiously, the highest k_{cat}/K_m^{ADP} of RM-CK was at 15 °C. Though at both pHs, k_{cat}/K_m^{ADP} of all three enzymes decreased as temperature decreased, the decrease in catalytic efficiency of M1-CK seemed to be the most gentle.

Biochemical kinetic properties of M1-CK were quite different from those of RM- and M3-CK (Figure 4 and Table 2). K_m^{PCr}, K_m^{ADP}, and k_{cat} of M1-CK varied least at different pHs and temperatures. K_m^{PCr} and K_m^{ADP} of M3-CK were all higher than those of the RM-CK under all pH and temperature conditions in the present study. Especially at pH 7.1, at 5 °C, K_m^{PCr} of M3-CK was more than 3.5 folds higher than K_m^{PCr} of RM- and M1-CK. Since K_m^{ADP} of all three enzymes were relatively stable, and all k_{cat}/K_m^{ADP} were much higher than all k_{cat}/K_m^{PCr} in all three enzymes, ADP binding did not seem to be the rate limiting factor in M-CK catalysis

at different pHs and temperatures (Table 2). When comparing the highest k_{cat}/K_m^{PCr} at pH 7.1, 25 °C to the lowest at pH 8.0, 5 °C, of all three enzymes, the decrease in k_{cat}/K_m^{PCr} were 9.4 folds in M1-CK, 15.9 folds in RM-CK, and 27.2 folds in M3-CK.

Activation energy of catalysis (*Ea*) was calculated using the *Arrhenius* equation (Lonhienne *et al.*, 2000),

$$k_{cat} = \mathrm{A}\, e^{-Ea\,/\,(\mathrm{RT})}$$

where $-Ea \bullet \mathrm{R}^{-1}$ is the slope of the Arrhenius plot when $\ln(k_{cat})$ is plotted as a function of T^{-1}. R is the gas constant (8.314 J • mole^{-1} • K^{-1}). *Ea* values (kJ • mole^{-1}) of M1-CK were the lowest among the three enzymes at both pHs (Table 2). *Ea* values of RM-CK were around 64 kJ • mole^{-1} at both pHs, which reflected that *Ea* of RM-CK was not affected by pH. However, *Ea* values of both M1- and M3-CK decreased when pH changed from 7.1 to 8.0. Therefore, M1- and M3-CK of the common carp were more affected by pH.

3.1.4. THERMAL STABILITY OF THE THREE M-CKS AND MONITORING OF THEIR SECONDARY STRUCTURE CONTENT WITH CD SPECTROSCOPY

A CD signal will be observed when a chromophore was placed in an asymmetric environment by virtue of the 3-dimensional structure adopted by the molecule, and CD spectrum was obtained when the dichroism is measured as a function of wavelength. In protein, different types of secondary structural elements such as α-helices, β-sheets, β-turns and random coil induced bands of distinctive shapes and magnitudes in the far-UV. For example, α-helix induced an intense positive band at 190 nm and a negative band at 208 and 220 nm. Therefore, the loss of CD signals either on addition of denaturing agents or by an increase in temperature could be used to provide quantitative estimates of the stability of the

folded state of the native protein (Kelly, *et al.*, 2005).

Comparisons of thermal stability of the three M-CKs showed that RM-CK was indeed the most thermal stable and M3-CK the least (Figure 5). Both RM- and M3-CK were rendered less thermal stable at pH 8.0, though by different extent. And, for M3-CK, even preincubation at 20 and 30 °C caused a significant decrease in its activity. At pH 7.1, after preincubation at 40 °C, M1-CK retained over 60% its highest activity, and at pH 8.0, 50%. Also, thermal stability of M1-CK did not differ significantly at both pHs.

The far UV CD spectra (200-250 nm) of RM-, M1-, and M3-CK were shown in Figure 6. It revealed that the secondary structures (α-helix and β-sheet) of RM- and M3-CK fluctuated significantly at both pHs and at different temperatures. While the overall CD profiles of M1-CK varied little from 40 to 5 °C, it only started to denature at 45 °C. Taken together, these results showed that M1-CK was more adaptive to the synchronized changes in body temperature and intracellular pH that occurred in the common carp.

3.2. ACTIVITY OF RABBIT MUSCLE-SPECIFIC CREATINE KINASE AT LOW TEMPERATURE BY MUTATION AT GLYCINE 268 TO ASPARAGINE 268

3.2.1. SECONDARY STRUCTURE ANALYSIS OF RM-CK AND ITS MUTANTS

In order to avoid disturbing the overall structures of the RM-CK, only the differences around the important Cysteine 283 between the RM-CK and M1-CK were compared (Figure 7a). At the N-terminal of cysteine 283, three variations between these two M-CKs, proline 270/glycine 270, glycine 268/aspartic acid 268 and alanine 267/histidine 267, were highly different in their residue types. All located outside or at the edge of any secondary structures. At the C-terminal of cysteine 283, the variations alanine 300/proline 300, histidine 301/lysine 301 and lysine 304/threoine 304 all participate in the α-helices that form the symmetric structures around the active site. Thus, they were not chosen to be mutated in this study.

CD spectroscopy was used to evaluate the overall secondary structures of the three RM-CK mutants (Figure 7b). The CD spectra of the RM-CK and three mutants were similar at pHs 7 and 8 and from 5 to 45 °C, thus only the CD spectra of the RM-CK and RM-CK G268N were shown. It seemed, below 35 ˚C, there was no significant difference in overall composition of α-helices and β-sheets between the wild-type and mutant enzymes.

3.2.2. SPECIFIC ACTIVITIES OF M-CKs AT DIFFERENT CONDITIONS

The *C. carpio* M1-CK behaved similarly to a psychrophilic enzyme when at a higher pH of 7.7, as shown in Figure 3. The specific activity of M1-CK at 15 °C, pH 7.7 was 65.3% its

highest activity at 30 °C, pH 7.1. At pH 8.0, M1-CK exhibited highest activity at 15 °C. The three RM-CK mutants showed different pH-temperature specific activity patterns comparing to the wild-type RM-CK (Figure 3). At pH 7.1, all three RM-CK mutants showed higher specific activities than the wild-type at 35 °C, with RM-CK G268N 23 ± 2%, and A267H and P270G more than 30 ± 7% higher than the wild-type. This was the first time that substitution of a single residue in M-CK had shown the enzyme to gain higher specific activity than its wild-type. At pH 7.7 and pH 8.0 at 10 °C; however, only RM-CK G268N exhibited 2 to 2.5-fold higher specific activity than the wild-type, comparable to *C. carpio* M1-CK. It was also interesting that the specific activities of RM-CK P270G showed steep decreases at 30 °C, but increase again at 25 °C at all the pHs examined. This was the first time that single change in residue in M-CK enabled the enzyme to gain higher specific activity than its wild-type. But, at pHs 7.7 and 8.0, only RM-CK G268N exhibited 6 to 8-folds higher specific activity than the wild-type at 10 °C, comparable to the *C. carpio* M1-CK (Figure 8; Figure 9).

Thermal inactivation assay of the M-CKs showed that wild-type RM-CK, RM-CK G268N and carp M1-CK were inactivated at the same temperature range around 40 °C at pH 7.1; however, the inactivation temperature of M1-CK N268G at pH 7.1 was below 40 °C (Figure 10). At pH 8.0, the thermal stability of M1-CK and RM-CK G268N actually improved, whereas wild-type RM-CK and wild-type M1-CK N268G started to lose their activity when the 30 minute pre-assay heat treatment temperature was above 20 °C. These results suggested that N268 contributes significantly to the thermal stability of M1-CK and RM-CK G268N.

3.2.3. KINETIC ANALYSIS OF RM-CK AND ITS MUTANTS

The k_{cat}/K_m^{Pcr} and k_{cat}/K_m^{ADP} of RM-CK mutants were shown in Figure 11. The K_m^{PCr} of RM-CK A267H was much higher than other enzymes at pH 7.1. The K_m^{PCr} of RM-CK G268N was lower than RM-CK in all conditions except at pH 7.1, at 15 °C. The K_m^{PCr} of M1-CK remained stable at pH 8.0. The K_m^{ADP} of RM-CK G268N was not higher than RM-CK in all conditions. At the same time, the K_m^{ADP} of M1-CK remained stable at both pHs. Comparing to that of RM-CK, the K_m^{ADP} of RM-CK G268N was changed less between temperatures at pH 8.0. Stable K_m^{ADP} at low temperature indicated the similar affinity of ADP at different temperatures.

The k_{cat} of RM-CK decreased while temperature decreased to 5 °C at both pH 7.1 and 8.0 (Table 3). Although all the other enzymes exhibited the same trends, the k_{cat} of M1-CK decreased less. The k_{cat} of RM-CK G268N and P270G, as M1-CK, decreased less at temperature higher than 15 °C.

To summarize k_{cat} of mutant enzymes changed less correlated well to the higher activity at low temperatures, the values of k_{cat}/K_m of RM-CK G268N were higher than RM-CK at 5 °C, at all pHs (Figure 11a-d). The values of RM-CK P270G decreased less at pH 8.0. The catalytic properties of RM-CK G268N and P270G were similar to those of the M1-CK at pH 8.0, at low temperature.

Both K_m^{Pcr} and K_m^{ADP} values of RM-CK G268N were similar to those of the wild-type RM-CK at pH 7.1 and pH 8.0 and from 35 °C to 15 °C (Figure 12 a-d). However, both K_m^{Pcr} and K_m^{ADP} of M1-CK N268G fluctuated more in comparison with those of M1-CK, RM-CK and RM-CK G268N. The k_{cat} values of RM-CK G268N were also similar to those of the wild-type (Figure 12 e-f).

3.2.4. THE CRYSTAL STRUCTURE OF RM-CK G268N

The crystal structure of RM-CK G268N, solved by molecular replacement using RM-CK as model and refined to 3.3 Å resolution (Table 4), consists of two independent RM-CK G268N dimers contained in the crystal asymmetric unit. The backbone structure of RM-CK G268N was similar to the RM-CK (2crk). The backbone structures of two models between glycine 268 (Aspartic acid 268) to cysteine 283 were almost overlapped. The Cα group of cysteine 283 in RM-CK G268N was 0.2 Å shorter than RM-CK (Figure 13a). The secondary structure of RM-CK G268N was identical to RM-CK. The Aspartic acid 268 was located at the outside of molecule, while the side chain of the residue was point to outside (Figure 13b, c). In the modeling structure of RM-CK, consulted with the previous CK structure studies, valine 72, arginine 130, arginine 132, glutamic acid 232, arginine 236 and arginine 320 were located to set the boundary of active site (Figure 13d, e). The distances of each selected residue pairs and the distances between the thiol group of cysteine 283 and Cα of selected residues were calculated (Table 5a). Five of these residues were closer to the thiol group of cysteine 283 in RM-CK G268N than in RM-CK. The distances of most residue pairs decreased in the RM-CK G268N, that is, a smaller active site pocket formed.

The distances between 6 chosen residues were calculated to compare the sizes of the active site pockets (Table 5b). The distances between the creatine binding residues (valine 72 and glutamic acid 232) to the nucleotide binding residues (arginine 132, 236, 292 and 320) are closer in RM-CK G268N than in RM-CK. The distance is 0.4 Å shorter from valine 72 to arginine 292 in RM-CK G268N than in RM-CK, and the distance is 0.2 Å shorter from glutamic acid 232 to arginine 236 in RM-CK G268N (Figure 13d, e). The smaller active site pocket of RM-CK G268N may enable this mutant to function at low temperature.

3.2.5. SUBSTRATE SUBSTITUTION OF CKS

If the active sites of M1-CK and RM-CK G268N have become smaller, a smaller substrate, 2'-deoxyadenosine-5'-diphosphate (dADP), which has been reported to react with PCr by CK catalysis, the activities of M1-CK and RM-CK G268N should be higher than RM-CK. RM-CK, RM-CK mutants and M1-CK exhibited similar activities at pH 7.1, at temperature over 15 °C (Figure 14a). At this pH, the activities of RM-CK, RM-CK A267H and RM-CK G268N with dADP substrate were higher than those of ADP at 5 °C.

At pH 7.4, the activities of RM-CK at 35 and 25 °C were lower than its activities at the same temperature at pH 7.1, which the activities of M1-CK and RM-CK G268N were very similar to those at pH 7.1 (Figure 14b).

At pH 7.7, the highest activity of RM-CK was at 15 °C and the activities of RM-CK G268N at temperature higher than 15 °C were still similar to those at pH 7.1 and 7.4 (Figure 14c). The activities of M1-CK and RM-CK G268N at 10 and 5 °C were relatively higher than RM-CK. M1-CK had highest activity at 25 °C at this pH, and its activity at low temperature was higher than RM-CK and the rabbit mutants at this pH.

The M1-CK showed high activities at 25 and 15 °C, and maintained relatively higher activities at 10 and 5 °C than RM-CK and the rabbit mutant (Figure 14d). The activities of RM-CK G268N at temperature higher than 15 °C were stable.

To summarize, the activities of RM-CK, RM-CK G268N and M1-CK with dADP as one of the substrate, at low pH, could maintain their activities at high temperature better than 15 °C. However, at high pH, only M1-CK and RM-CK G268N could maintain stable activities at temperature above 15 °C. Data of the specific activities of all enzymes were shown in Table 6.

3.3. ACTIVITY OF MUSCLE FORM CREATINE KINASE AT LOW TEMPERATURE MAY DEPEND ON HYDROPHILICITY OF SIDE CHAIN OF RESIDUE 268

3.3.1. MONOPHOSPHORYLATED ENZYMES WERE DETECTED

The fragments from residue 267 to 292 was digested with trypsin and since the digestion site is conserved between RM-CK and M1-CK, the exact molecular weight is listed in Table 8. There are three threoine which could be phosphorylated in this fragment. The phosphorylated fragment molecule weight is also presented at in Table 8. The mass spectra shows there are symbol peaks which correspond to monophosphorylated fragments at the exact m/z axis (Figure 15). All ten enzymes are identified with monophosphorylated form.

3.3.2. RESIDUE 268 OF M1-CK AND ITS MUTANTS WITH HYDROPHILIC SIDE CHAIN MAINTAINED BETTER SECONDARY STRUCTURE CONFORMATION AT 15 °C

The CD spectrum of each enzyme was plotted at different temperature and pH. Most of the secondary structures of CK and mutants were not disrupted during temperature change, except M1-CK N268L at 15 °C, pH 8.0. For RM-CK and its mutants, the overall secondary structure of RM-CK G268K changed except at 35 °C, pH 7.1. The other mutants of RM-CK were not affected by temperature and pH. In M1-CK and its mutants, the M1-CK N268L could maintain its conformation at 35 °C, but not at 15 °C, at both pH 7.1 and 8.0. Therefore, according to the RM-CK and M1-CK mutants' spectra, hydrophilic side chain of residue 268 might help the secondary structure stable in a functional conformation (Figure 16).

3.3.3. POLAR SIDE CHAINS OF RESIDUE 268 OF M-CK MAINTAIN ACTIVITY AT LOW TEMPERATURE

RM-CK and M1-CK residues 268 were mutated to asparagine, glycine, aspartate, lysine and leucine, individually. RM-CK G268N, similar to M1-CK, maintained its activity at 10 °C, pH 7.7 and 8.0 as show in Figure 3 and Figure 9. At 40 and 35 °C, pH 7.1, RM-CK G268N exhibited higher activity (391.88 ± 27.06; 436.53 ± 7.08) than RM-CK (292.80 ± 17.99; 352.76 ± 23.86), RM-CK G268D (381.83 ± 25.31; 401.19 ± 3.72) and G268K (388.68 ± 32.92; 407.55 ± 1.05) (Figure 17a). Otherwise, RM-CK G268D and G268K exhibited higher activities than native form RM-CK and RM-CK G268L at all pHs and temperatures. The activity of RM-CK G268N was close to RM-CK, but lower than RM-CK G268D and G268K except at low temperature (10 °C) and high pH (pH 7.7 and 8.0)(RM-CK G268N/RM-CK G268D/RM-CK G268K: 82.77 ± 10.62/51.90 ± 4.90/66.11 ± 6.37; 74.15 ± 7.61/28.04 ± 1.97/33.71 ± 1.85)(Figure 17a-d).

M1-CK N268G showed lower activity at 15 °C, pH 7.7 and 8.0 than M1-CK native form (Wu *et al.*, 2011). M1-CK N268L was similar to M1-CK N268G. At 25 °C, M1-CK N268L showed higher activity (pH 7.1, 7.4, 7.7 and 8.0: 340.92 ± 6.73; 197.43 ± 7.75; 133.68 ± 3.99; 68.04 ± 2.89, respectively) than M1-CK N268D (pH 7.1, 7.4, 7.7 and 8.0: 266.81 ± 18.43; 167.99 ± 13.26; 100.18 ± 9.45; 48.10 ± 6.38, respectively) and N268K (pH 7.1, 7.4, 7.7 and 8.0: 208.12 ± 20.06; 163.49 ± 14.10; 91.82 ± 7.70; 34.06 ± 3.22, respectively). Furthermore, M1-CK N268L had higher activity (89.37 ± 10.93) than M1-CK N268D (89.24 ± 5.18) and N268K (64.94 ± 8.09) did at 35 °C, pH 8.0 (Figure 17h). Otherwise, activities of M1-CK N268L were lower than other two mutants. However, M1-CK N268D and N268K could recover the activities at 15 °C of all pH and 10 °C, pH 7.7 (Figure 17e-h).

At high temperature, 35 or 30 °C, polar side chain of residue 268 of M-CK increased their enzyme activity over non-polar ones. A polar side chain of residue 268 of M-CK also maintained higher activity than non-polar ones at low temperature and high pH.

3.3.4. RM-CK G268D AND M1-CK N268D WERE DIFFERENT FROM THE OTHER ENZYMES IN KINETIC PARAMETERS

The kinetic parameters, K_m^{ADP}, K_m^{PCr}, k_{cat}, of each enzyme were measured at 35, 25, 15, 5 °C, pH 7.1 and 8.0. K_m^{ADP} of RM-CK and its mutants were higher at 35 °C than at 15 °C in both pH 7.1 and 8.0. The K_m^{ADP} of RM-CK G268D (0.77 ± 0.16) is much lower than RM-CK (1.43 ± 0.11) at 35 °C, pH 7.1. The same property can be observed at 15 °C, pH 7.1 (RM-CK / RM-CK G268D: 0.37 ± 0.04 / 0.23 ± 0.03). However, K_m^{ADP} of RM-CK G268N (1.36 ± 0.14; 0.33 ± 0.02), RM-CK G268K (1.53 ± 0.18; 0.37 ± 0.04) and RM-CK G268L (1.53 ± 0.18; 0.29 ± 0.03) are not significantly different with RM-CK (1.43 ± 0.11; 0.37 ± 0.04) at 35 and 15 °C, pH 7.1. At pH 7.1, glycine replaced by aspartic acid at residue 268 of RM-CK would decrease the K_m^{ADP} (Figure 18a). At pH 8.0, RM-CK G268K maintained its K_m^{ADP} when temperature decreased (Figure 18b).

K_m^{ADP} of M1-CK N268D similar to that of M1-CK at 35 to 15 °C, pH 7.1 and 8.0, except at 15 °C, pH 8.0 (Figure 18a, b). RM-CK N268D maintained its K_m^{ADP} at pH 8.0 when temperature decreased. K_m^{ADP} of the other M1-CK mutants would maintain their K_m^{ADP} between different temperatures, both at pH 7.1 and 8.0 (Figure 18a, b).

K_m^{PCr} of creatine kinase was highly influenced by ambient pH. K_m^{PCr} of RM-CK, M1-CK and their mutants increased from 35 to 15 °C, at pH 7.1 and decreased at pH 8.0.

During the temperature decrease from 35 to 15 °C, K_m^{PCr} of RM-CK G268D increased

most at pH 7.1 (3.48 ± 0.46 to 44.25 ± 0.52). At the same time, it also decreased most at pH 8.0 (20.12 ± 1.14 to 7.82 ± 0.44). Except RM-CK G268D, K_m^{PCr} of RM-CK and mutants were not significantly different at 35 and 15 °C, pH 8.0 (Figure 18c, d).

At 35 °C, K_m^{PCr} of M1-CK N268G (7.10 ± 0.62), N268D (6.45 ± 0.37) and N268K (8.59 ± 0.53) are similar to M1-CK (6.75 ± 0.16) but lower than M1-CK N268G (15.21 ± 1.26) at pH 7.1. K_m^{PCr} of M1-CK N268D is almost identical as M1-CK N268G at pH 8.0. However, at pH8.0, K_m^{PCr} of all M1-CK mutants are lower than M1-CK at 15 °C and close to M1-CK N268G. The K_m^{PCr} of M1-CK N268K was extremely high at 35 °C, pH 8.0. Otherwise, K_m^{PCr} of all other M1-CK mutants were close to M1-CK at 35 °C, pH 8.0 (Figure 18c, d).

k_{cat} of RM-CK G268D and G268K are much higher than other RM-CK mutants at 35 °C, pH 8.0. At the other conditions, there is no significant difference between different enzymes. For the M1-CK and mutants, k_{cat} of M1-CK N268D, N268K and N268L are higher than M1-CK at 35 °C, pH 7.1. k_{cat} of M1-CK N268G, N268D and N268K are different from M1-CK at 35 °C, pH 8.0. But, all M1-CK mutants were not significantly different from M1-CK at 15 °C, both pH 7.1 and 8.0 (Figure 18e, f).

3.3.5. POLAR SIDE CHAIN OF RESIDUE 268 MODIFIED THE SOLVENT ACCESSIBLE SURFACE OF ENZYME TO DECREASE THE DISTANCE BETWEEN SUBSTRATES IN ACTIVE SITE

The RM-CK G268N 3-D structures were analyzed using molecular modeling program. The distance between Cα from aspartic acid 268 to cysteine 283 in RM-CK G268N was shorter than from glycine 268 to cysteine 283 in RM-CK as show in Figure 13. Then, the solvent accessible surface (SAS) area difference between RM-CK and RM-CK G268N was

evaluated (Table 9). The SAS of RM-CK G268N was smaller than that of RM-CK (Figure 19a).

Comparing the back surface of RM-CK and RM-CK G268N, two clusters of hydrophilic residues between glutamic acid 160 to lysine 172 and lysine 177 to glutamic acid 183 shift closer to aspartic acid 268 in RM-CK G268N (Figure 19b). The hydrophilic clusters increase water molecule interaction with the enzyme. The distance between Cα of aspartic acid 268 to threoine 103, arginine 148, arginine 152, glutamic acid 160, aspartic acid 163, lysine 170, lysine 172, lysine 177, serine 178 and glutamic acid 183 were calculated in Table 9. The distance between Cα from aspartic acid 268 to threoine 103 in RM-CK G268N is the same as from glycine 268 to threoine 103 in RM-CK. The distances between Cα from aspartic acid 268 to arginine 148 and arginine 152 in RM-CK G268N are shorter than from glycine 268 to arginine 148 and arginine 152 in RM-CK. However, the other highly solvent accessible residues were move closer to aspartic acid 268 in RM-CK G268N than to glycine 268 in RM-CK.

The solvent accessible surface area of each residue is shown in Table 9. Most SAS of residues are reduced due to the whole molecule becomes smaller in RM-CK G268N. The hydrophobicity indexes around residue 268 of RM-CK G268N, G268D and G268K are more hydrophilic than RM-CK for 0.6 score, and RM-CK G268L for 1.5 score (Figure 20a). M1-CK, M1-CK N268D and M1-CK N268K are more hydrophilic than M1-CK N268G for 0.7 score, and M1-CK N268L for 1.5 score around residue 268 (Figure 20b). The hydrophobicity of the surface of CK reduces to enhance the compactness of the active site.

CHAPTER 4. DISCUSSION

4.1. "CARP M1-CK", AN ENZYME FOR ALL SEASONS

Environmental temperature directly influences the extra- and intracellular pH of poikilothermal fishes, subsequently intracellular pH influences the functions of all enzymes (White and Somero, 1982). In this study, the possible physiological function of M1-, M2-, and M3-CK was studied by comparing their biochemical kinetic features with RM-CK at pHs that resemble the variation in intracellular pHs in the common carp at different temperatures (Figure 3). At pH 8.0, 15 °C, only M1-CK was able to maintain its specific activity at more than 50% (156 U) its highest specific activity (287 U, at pH 7.1, 30 °C), while specific activity of M3-CK decreased to 25 U. These results suggested that, in carp muscle, M1-CK alone could play a major role in maintaining energy homeostasis at various water temperatures.

In earlier studies, no change was observed in the percentages of mRNA and protein content of the three subisoforms of carp M-CKs under different water temperatures, thus, the adaptive strategy of the M-CK enzymes towards variable environmental temperatures would likely be at the enzyme activity level (Sun, *et al.,* 2002; Andrew, *et al.,* 2004). In a previous study, M3-CK homodimer could not be detected at temperature above 20 °C (Sun, *et al.,* 2002), pH 7.4, yet in the present study, in the modified assay buffer, at pHs from 7.1 to 7.7, and at temperatures below 30 °C, specific activities of M3-CK are comparable to those of RM-CK (Wu, *et al.*, 2008). This discrepancy could be due to that the previous dimerization experiment of M3-CK was carried out in PBS which was devoid of Mg^{2+}, while the present assay buffer contained 10 mM $MgCl_2$ and 1 mM DTT and both might help in the stabilization of M3-CK dimer at temperatures above 20 °C. Specific activities of M1- and M3-CK are

much higher in the present modified buffer than when the commercial M-CK assay kit was used in our previous studies (Sun, *et al.,* 1998).

At low temperature, k_{cat} in psychrophilic enzyme was generally higher than mesophilic enzyme, and activation energy of catalysis (*Ea*) of psychrophilic enzyme decreased to favor k_{cat} (Lonhienne, *et al.,* 2000). *Ea* values of M1-CK were the lowest among the three enzymes both at pH 7.1 and 8.0, furthermore, *Ea* value of M1-CK at pH 8.0 was lower than that at pH 7.1 (Figure 4, Table 2). This unique functional property of M1-CK, relatively stable K_m^{PCr} and K_m^{ADP}, and lower *Ea*, suggests that M1-CK had evolved to become an enzyme that could function at pHs between 7 to 8 and at temperatures from 35 to 5 °C (Cook, *et al.,* 1981).

An advantage of CD spectroscopy was that it enables the monitoring of dynamic changes in solution in the secondary structures of RM-, M1-, and M3-CK at different pHs and temperatures (Kelly, *et al.,* 2005). Changes in secondary structures of M1-CK at the pHs and temperatures under studied were much less than in the cases of RM- and M3-CK. For M3-CK, at 30 °C, its CD spectra at both pHs show that its secondary structures started to deviate from the 25 °C profile. Crystal structures of human and RM-CKs had been determined (Rao, *et al.,* 1998; Shen, *et al.,* 2001; Ohren, *et al.,* 2007). The high-resolution RM-CK structure shows that aspartic acid 55 in the RM-CK-ADP subunit formed a hydrogen bond to the side chain of histidine 7 in the adjacent RM-CK-TSA subunit, while aspartic acid 55 from the RM-CK-TSA subunit formed a hydrogen bond to the backbone N atom of proline 2 within the same chain (Ohren, *et al.,* 2007). It had been proposed that the loss of this hydrogen bond was sufficient to cause dissociation of the dimer. In the case of M3-CK, both the proline 2 and histidine 7 are absent in the N-terminal, thus, it might be the reason for the thermal instability of this enzyme (Sun, *et al*., 1998).

It had been suggested that a 16 °C range of temperatures could not be covered by one metabolic profile (Guderley, 1990). Indeed, myofibrillar heavy chain (MHC) isoform expression can be modified by thermal acclimation, and production of biochemically different myofibrillar ATPase in white muscle could response to environmental temperature (Johnston, *et al.,* 1990; Gerlach, *et al.,* 1990). More recently, cold induced gene expression had been found to include acyl-CoA △9-desaturase, RNA polymerase II activators, RNA helicase, TCP1 chaperonins, and a large group of genes involved in ubiquitin-dependent protein catabolism and proteasomal function (Gracey, *et al.,* 2004). In the case of carp M-CKs, a study using 2-D gel on cold acclimation and expression of soluble proteins in carp skeletal muscle revealed a downregulation of expression of M2- and M3-CK, and an increased accumulation of their fragments (McLean, *et al.,* 2007). Taken together, M3-CK was thermal unstable as well as subject to ubiquitination and proteolytic fragmentation at low temperature. And, since M2-CK was much less active, we propose that M1-CK was the only one that functioned well at pH 7 to 8 and from 35 to 5 °C. Of course, post-translational regulation of the carp M-CKs at different temperatures could not be ruled out, but at least it was not observed in the 2-D gel studies (McLean, *et al.,* 2007).

In this study, the biochemical characteristics, thermal stability, and CD spectroscopy analyses of two muscle-specific subisoforms of CK of the poikilothermal common carp, and RM-CK of an endothermic animal were compared. In conclusion, the M1-CK is the only one that functions well at pHs from 7 to 8, and from 35 to 0 °C. And, since selection pressure on each carp muscle-specific subisoform would be less than if there were only one muscle-specific isoform, M1-CK subisoform seemed to be the only one that had evolved to become adaptive to the synchronized changes in body temperature and intracellular pH that occur in the common carp.

4.2. ONE RESIDUE CHANGE EXTENDS CK ACTIVITY TEMPERATURE RANGE

The temperature of the habitat of the common carp varied from 5 °C to >35 °C and its extracellular and intracellular fluid pH varied inversely with body temperature (Yancey and Somero, 1978; Hwang and Lin, 2002; Stecyk and Farrell, 2006). Previously, it was suggested that a 16 °C range of temperature cannot be executed by one metabolic profile (Guderley, 1990). However, a more recent analysis of the three carp muscle-specific creatine kinase isoforms revealed that only M1-CK functions well at pHs from 7 to 8 and from 0 °C to 35 °C (Wu, *et al.*, 2008).

Biochemical analyses indicated that the K_m and k_{cat} of psychrophilic enzymes from Antarctic and Arctic organisms were higher than those of their mesophilic or thermophilic homologous due to lower activation energy (E_a) (Marshall, 1997). The molecular expectation of enzyme function at low temperature had been examined by comparing homologous enzymes from organisms whose habitats spanned a wide range of temperatures and it was found that the thermal denaturation temperature of cold-adapted enzymes was lower than that in mesophilic homologous which suggested higher flexibility in cold-adapted enzymes (Feller and Gerday, 1997; Smalas, *et al.*, 2000; Hoyoux, *et al.*, 2004; Bae and Phillips, 2004).

High flexibility was accompanied by a trade-off in stability, resulting in heat lability, and in the few cases studied, cold lability (Siddiqui and Cavicchioli, 2006). For RM-CK, a single mutation in D54G decreased the midpoint temperature of thermal inactivation by 16 °C and resulted in a 79% decrease in its activity at 25 °C, inferring that increase in flexibility of the protein in turn caused partial unfolding of the protein (Feng, *et al.*, 2007). However, in the present study, CD spectra, specific activity and thermal stability showed that M1-CK and RM-CK G286N were both flexible and yet thermally stable and function from pH 7 to pH 8 and from 35 to 10 °C. The residue change in the RM-CK mutant was located at the surface of

the molecule, thus intracellular pH change might be an important factor affecting flexibility of the protein. These kinetic properties suggested that in addition to flexibility there might be other possible factors allowing M1-CK and RM-CK G286N to function over a broad range of temperatures. The crystal structure of RM-CK G268N revealed one such possible factor.

RM-CK had been cloned and its crystal structure, 2crk, had been resolved (Putney, *et al.*, 1984; Rao, *et al.*, 1998). The cysteine 283 residue is important in keeping creatine anchored and positioned for nucleophilic attack on the -phosphorus of MgATP within a SN2 type reaction (Milner-White and Watts, 1971; Lahiri, *et al.*, 2002). Arginine 96, arginine 132, arginine 236, arginine 292 and arginine 341 are correlated to nucleotide binding, and valine 72, glutamic acid 232, glutamic acid 241 and aspartic acid 326 were correlated to creatine binding (Figure 21) (Wood, *et al.*, 1998; Cantwell, *et al.*, 2001; Tanaka and Suzuki, 2004; Feng, *et al.*, 2007; Uda, *et al.*, 2009). The crystal structure of RM-CK G268N showed that the distances of these residues to the target atoms of the substrates were all shorter in the mutant than in the wild-type, and thus might improve stabilization of the substrates docking in the active site in the mutant. Further modeling showed that the thiol of cysteine 283 and the hydroxyl of serine 285 of RM-CK G268N moved 0.44 and 0.77 Å, respectively, away from the active site. The distance between the thiol of cysteine 283 and the hydroxyl of serine 285 lengthened by 0.51 Å in RM-CK G268N (Figure 22a, b).

In RM-CK, it has been shown that serine 285 and proline 284 decrease the *pKa* of cysteine 283 thiol by around 2.2 pH units, thus, at neutral pH, cysteine 283-anion is important in maintaining the linear alignment necessary for associative inline transfer of a phosphoryl group (Wang, *et al.*, 2001; Wang, *et al.,* 2006). In the present work, the apparent pK_a of RM-CK, RM-CK G268N and M1-CK at pH 7.1 and pH 8.0 and from 35 °C to 15 °C were calculated based on the kinetic data, and pK_a values of RM-CK G268N were higher than

those of RM-CK (Figure 22c) (He, *et al.*, 2007). The most striking feature is that at 15 °C, the pK_a values of RM-CK G268N and M1-CK are almost identical at pH 7.7, as well as 0.35 pH units higher than that of RM-CK. In the case of carp M1-CK, as ambient temperature decreased and pH of intracellular fluid increased above pH 7.7, deprotonation of cysteine 283 thiol became less likely. At pH 8.0, the kinetic mechanism appeared rapid equilibrium random in both directions on the basis of both initial velocity studies (Morrison and James, 1965; Cook, *et al.*, 1981).

According to Cook *et al*, 1981, in substrate phosphocreatine binding, the rate constants k_8/k_5 ratios were 2.5 at 25 °C and 9.3 at 12 °C, k_8 being the rate constant of phosphocreatine released from E-MgADP-phosphocreatine and k_5 being that of proton release to H_2O from EH-MgADP-phosphocreatine, and k_5 appears to be highly temperature sensitive (Cook, *et al.*, 1981). In the reverse reaction, the rate constants k_3/k_2 ratios are 4.0 at 25 °C and 11.3 at 12 °C, k_3 being the rate constant of product creatine release from EH-MgADP-phosphocreatine, and k_2 that of phosphocreatine released from EH-MgADP-phosphocreatine, and k_2 was more temperature dependent than k_3. Thus, at low temperature, phosphocreatine and proton were stickier, and if k_3 and k_8 are less temperature sensitive and change little, k_2 and k_5 should decrease. The outcome, then, also would favor the release of phosphocreatine or creatine from the active site (Cook, *et al.*, 1981).

Assay with a smaller substrate, dADP, also showed that activities of RM-CK G268N from pH 7.4 to pH 8.0 were all higher than the wild-type though not as prominent as M1-CK. And the K_m^{dADP} of RM-CK G268N were all smaller than the wild-type in all conditions (Table 7). Thus one change in G268N in RM-CK indirectly caused a tiny decrease in the 3-D structures of the active site of RM-CK G268N, and this decrease also increased the apparent pK_a of the RM-CK G268N. Although M1-CK still functioned better from pH 7.1 to pH 8.0,

10 to 30 °C due to G268N as well as other changed in amino acid residues, it might still be concluded that one change in G268N at a non-critical part of the enzyme might make a significant contribution in causing RM-CK G268N and M1-CK to be more cold-adaptive. In the future, attempts will be made to change multiple sites such as G268N and A267H to see if CK enzymes could be made to function well across pHs 7 to 8 and from 35 to 5 °C; their 3-D structures will be studied.

4.3. COLD ACTIVATED FUNCTION OF M-CK RESIDUE 268

Comparing the activity of different M1-CK mutants at different conditions, M1-CK mutants with polar side chain amino acids in residue 268 might benefit their activity at 40 °C. M1-CK N268D and N268K could also maintain their activity as M1-CK, except at 10 °C, pH 7.1. Similar results were obtained with RM-CK mutants. Mutants with polar side chain at residue 268 showed higher activities than RM-CK at all conditions assayed. However, the RM-CK mutant with non-polar side chain at residue 268 showed much lower activities, comparison to those with polar ones. From the structure simulation analysis, the polar side chain of residue 268 located at the surface of the enzyme and in contact with water. The interaction between residue 268 of CK and H_2O might influence the conformation of the enzyme, especially the active site.

A charged group of amino acid side chain mainly interacted with charged molecules or atoms in the solution. However, the kinetic results of the M1-CK and RM-CK mutants demonstrated that the charge of side chain of residue 268 mainly affecting the K_m of enzyme. The RM-CK G268D had low K_m^{ADP} at 35 °C, pH 7.1. At the same time, it had higher K_m^{PCr} at 35 °C, pH 8.0. The RM-CK could prevent ADP binding at low pH and high temperature. It provided a higher affinity for PCr at high pH and high temperature, as RM-CK G268D, M1-CK N268D provides a higher ADP affinity at 15 °C, pH 8.0. The k_{cat} results of different mutants show that a polar side chain might increase or maintain the enzyme k_{cat} at different temperature. For RM-CK mutants, RM-CK G268D and G268K increased k_{cat} 2 folds at 35 °C, pH 8.0, as M1-CK N268D and N268K did at 35 °C, pH 7.1. However, a higher k_{cat} of M1-CK N268L at 35 °C, pH 7.1 might due to the nearby residues with positive charges.

Since the side chain of residue 268 of RM-CK located on the surface of molecule and pointing out to the solvent, the hydrophobicity of residue 268 might be the possible reason for

protein folding at low temperature. A hydrophilic side chain might reduce the hydrophobicity on the interface of protein and solvent (Timasheff, 1993; Folch, *et al.*, 2010). In previous studies, folding of CK was obviously influenced by the salt, organic solvent and denaturants (Saks, *et al.*, 1986; Yang, *et al.*, 1997; Huang, *et al.*, 2001; Du, *et al.*, 2006). Glycerol was one of the substances which reduced the RM-CK activity (Feng, *et al.*, 2008). It also reduced the formation of hydrogen bond between water molecules (Bhatnagar, *et al.*, 2007).

The experiment results of this study, show that in the evolution of enzyme from homeothermal animal to poikilothermal animal, are mutation in the primary structure at an appropriate position, even if this position locates on the surface of the protein molecule may be enough to enable enzymes to function at a board range of physiological temperature.

CHAPTER 5. CONCLUSION

Muscle form creatine kinase is one of the most important enzymes involving in the energy homeostasis of cellular function. Since CK has been studied for more than half century, as a key enzyme in physiological response, there are specific regulation mechanisms embed in its structure to satisfy the challenge of environmental change (McLeish and Kenyon, 2005). From previous studies, based on human, rabbit and chicken, abundant mitochondrial and muscle CK reaction mechanisms have been revealed (Lim, *et al.*, 2010). However, CK reaction at different temperature and at a narrower physiological pH range has not been studied even though psychrophilic, mesophilic and thermophilic enzymes have been studied in some detail (Debashish, 2005).

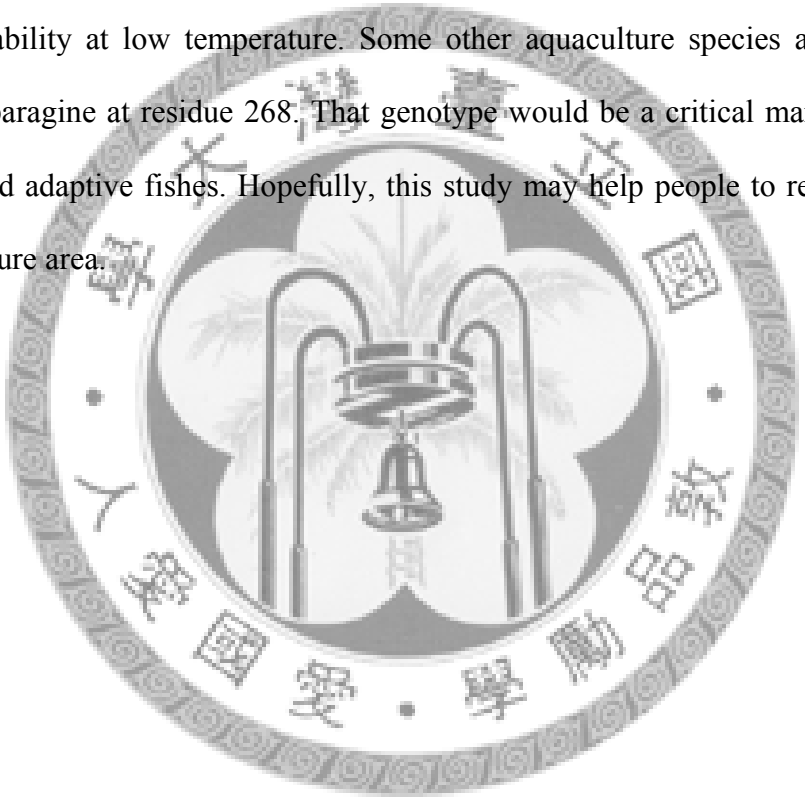
In this study, by analyzing the enzyme activity and thermodynamic characters of the wild-types RM-CK and M1-CK and their mutants, we show that RM-CK mutants and M1-CK and M1-CK mutants with a polar side chain at residue 268 exhibited higher activities at low temperature and at higher pH (Wu, *et al.*, 2008)

Crystal of RM-CK G268N was raised and by comparing the tertiary structure, a slight decrease in the pocket size of active site may be the reason to maintain M-CK activity at low temperature. And the smaller substrate, dADP, assays show the predicted result that a smaller active sites exist in a cold adaptive isozyme. (Wu, *et al.*, 2011a)

Protein modeling results demonstrate that the clustered hydrophilic motifs around residue 268 increase in surface area to increase the hydrophilicity to water bulk. Discuss with the hydrogen bond formation probability at low temperature may be the key to find out the criteria of a cold adapted enzyme. At the same time, this study offer a possible reason for the

enzyme evolution during low temperature with simply a site directed mutagenesis. There is a useful point in study the physiological and physicobiochemical properties of an energy homeostasis enzyme.

To sum up, we have offer a possible explanation for an energy homeostasis enzyme to function at a board range of physiological temperature by physicochemical and structural studies. Based on this study, some of biochemical strategies to prevent cold stress damage of aquaculture species are available recently. Carp M3-CK transgenic zebrafish could maintain its swimming ability at low temperature. Some other aquaculture species are also contain M-CK with asparagine at residue 268. That genotype would be a critical marker for genetic selection of cold adaptive fishes. Hopefully, this study may help people to reduce economic loss in aquaculture area.



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FIGURES

Figure 1.

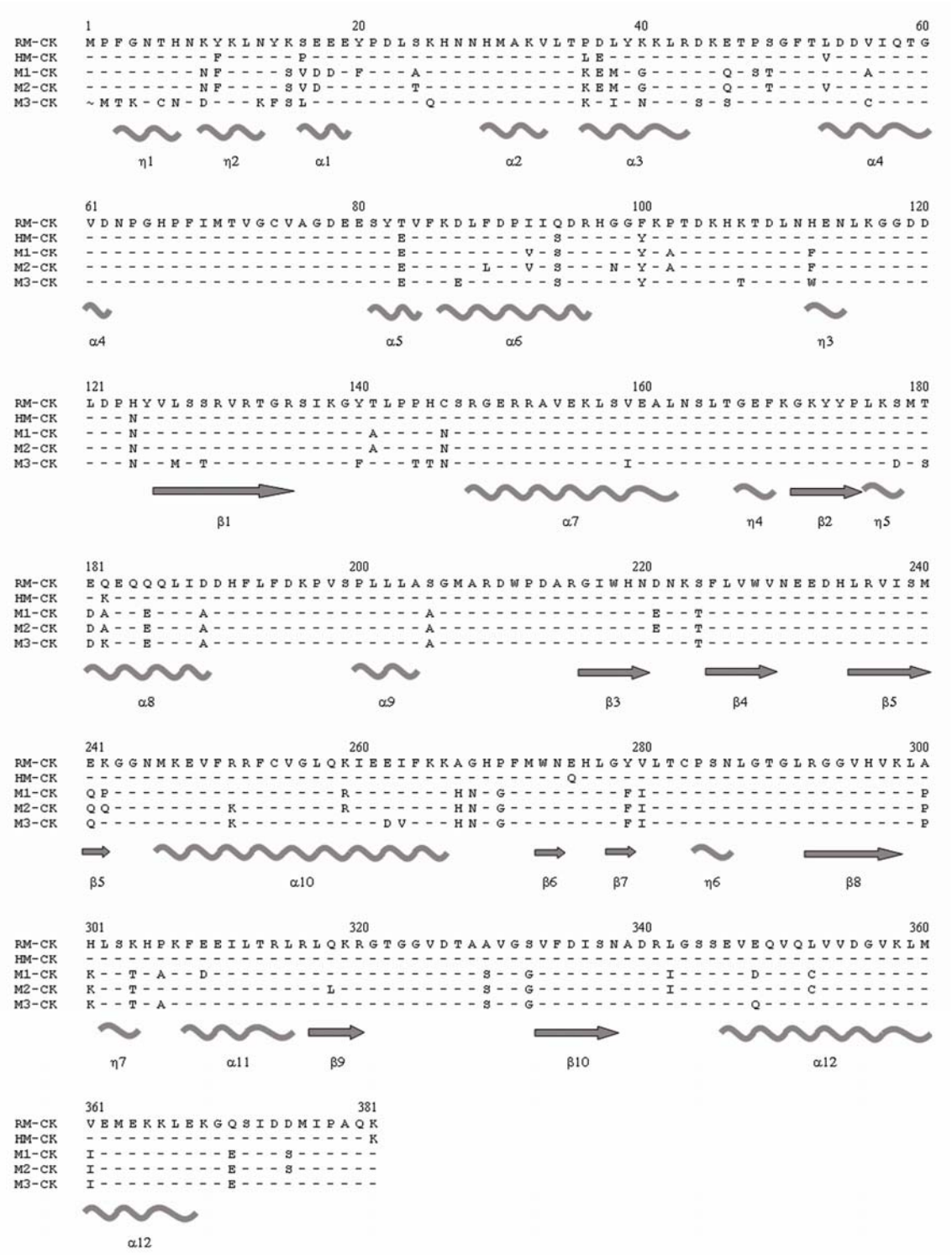
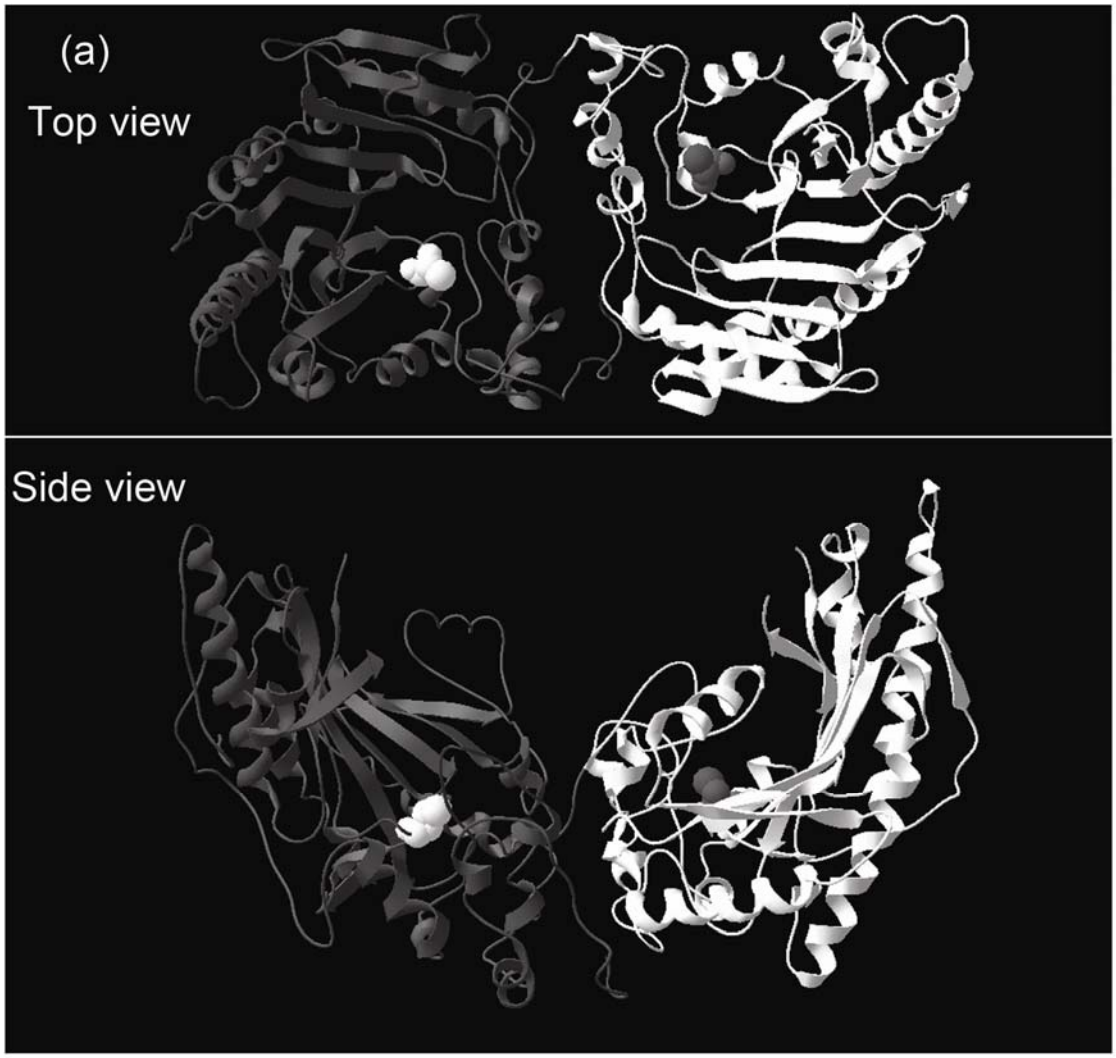
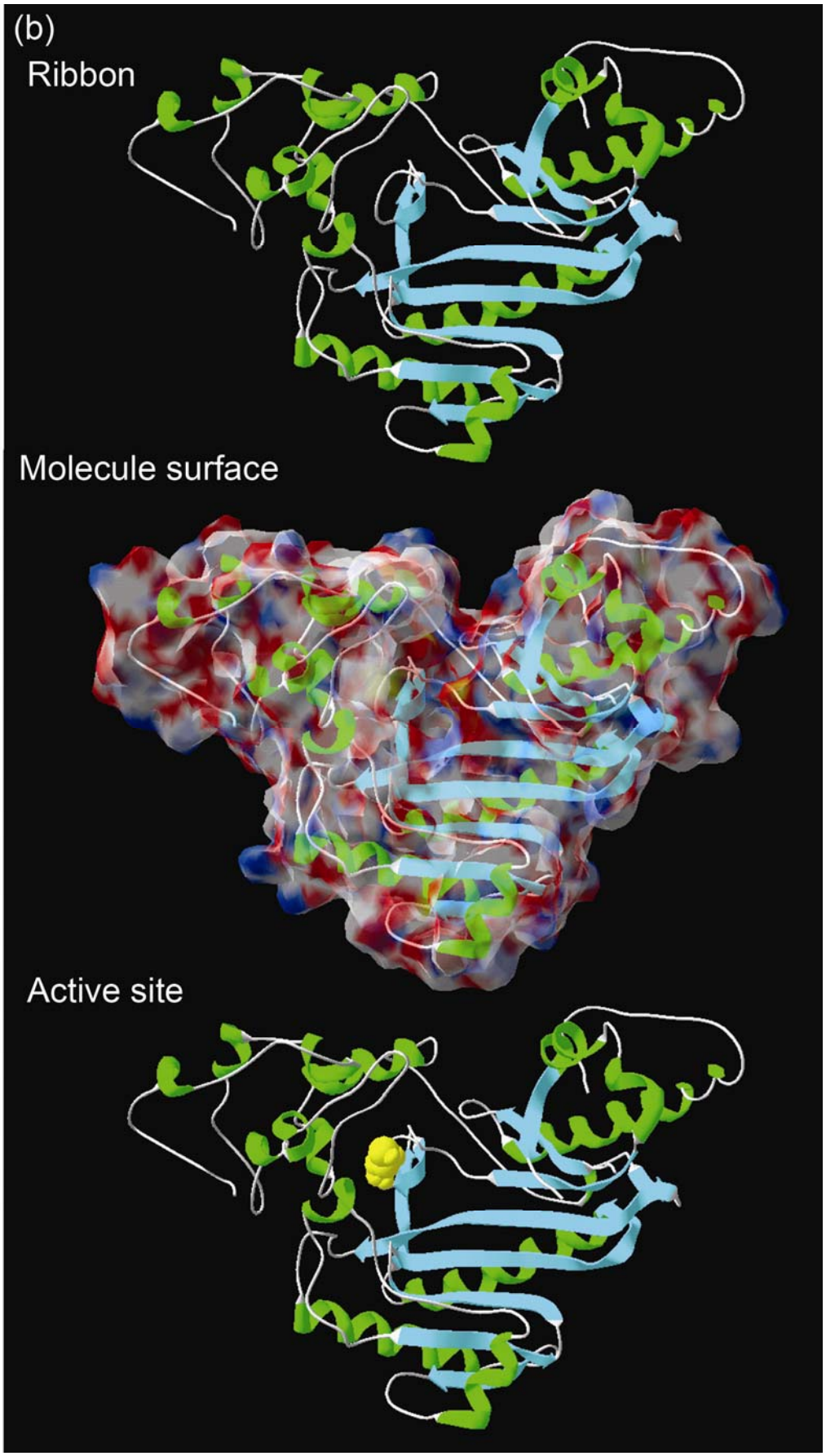


Figure 1. Sequences alignment of human, rabbit and carp M-CKs. The sequences

alignment of rabbit (RM-CK), human (HM-CK), carp M1-CK (M1-CK), carp M2-CK (M2-CK) and carp M3-CK (M3-CK). The secondary structure of M-CKs was presented below the sequences. Curves were α-helix with the number α1 to α12 and β-turns with number η1 to η7. Arrows were β-sheet with number β1 to β10.

Figure 2.




(b)
Ribbon
Molecule surface
Active site

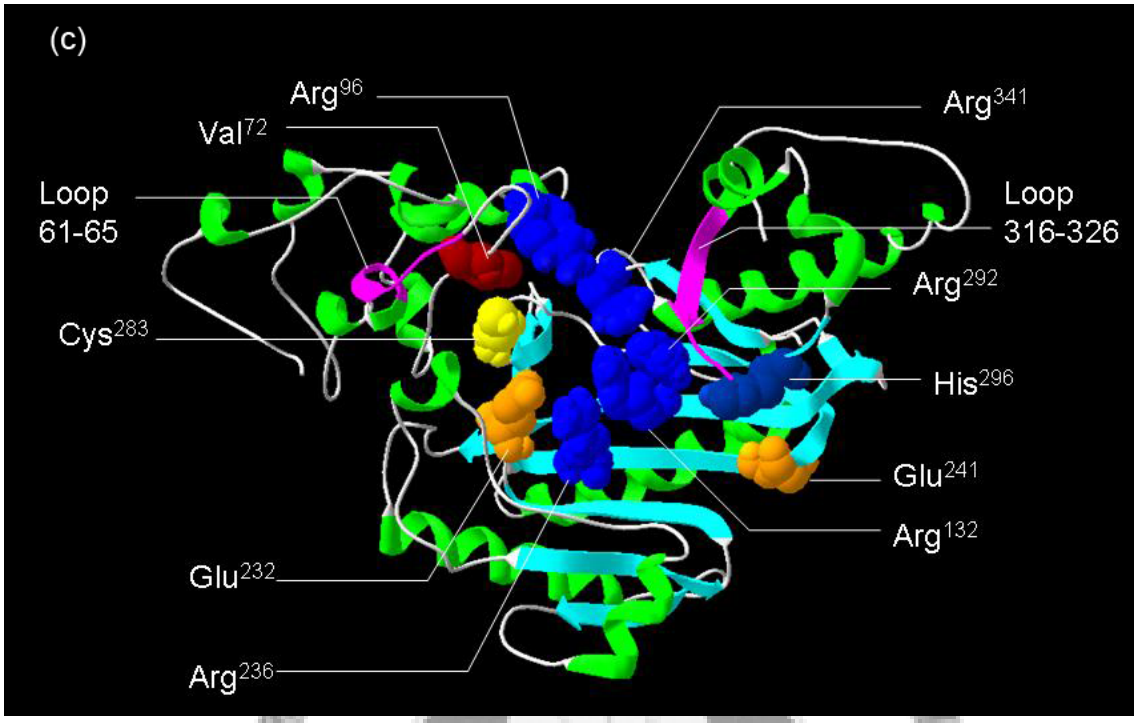


Figure 2. Molecular modeling of RM-CK. (a) Dimer form of RM-CK. Top view and side view of RM-CK dimmer with cysteine 283 located (CPK model). (b) Ribbon of RM-CK monomer. Secondary structures were represented in different color. Green: α-helix; blue: β-sheet. Molecule surface of RM-CK. Electronic potential was displayed. Red: negative charged; blue: positive charged. Active site of RM-CK. The yellow residue is cysteine 283. (c) All the residues involved in the substrate binding and reaction. Residues were labeled in the figure.

Figure 3.

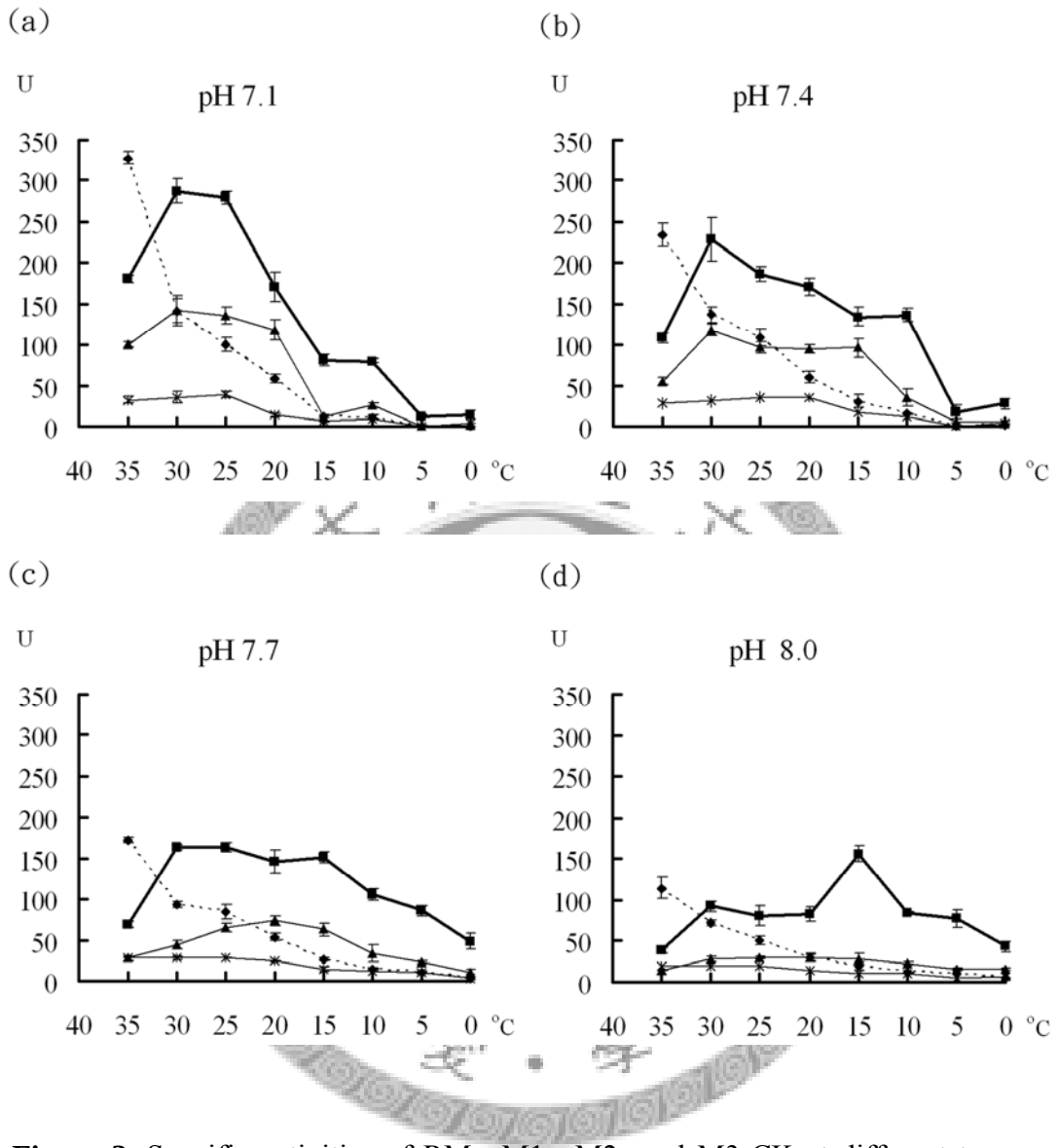


Figure 3. Specific activities of RM-, M1-, M2- and M3-CK at different temperatures and pHs. At least 3 replicates from three batches of recombinant protein preparation were conducted in each condition. The product Cr was reacted with α-naphthol and diacetyl, and the colour product absorbance was measured at 520 nm. The blank reaction without enzyme was subtracted from the absorption values. Buffer pH (a) pH 7.1, (b) pH 7.4, (c) pH 7.7, and (d) pH 8.0. ♦: RM-CK; ■: M1-CK; ∗: M2-CK; ▲: M3-CK. Error bars designate standard deviations. U is defined as 1 μmol of creatine formed per minute per mg of enzyme.

Figure 4.

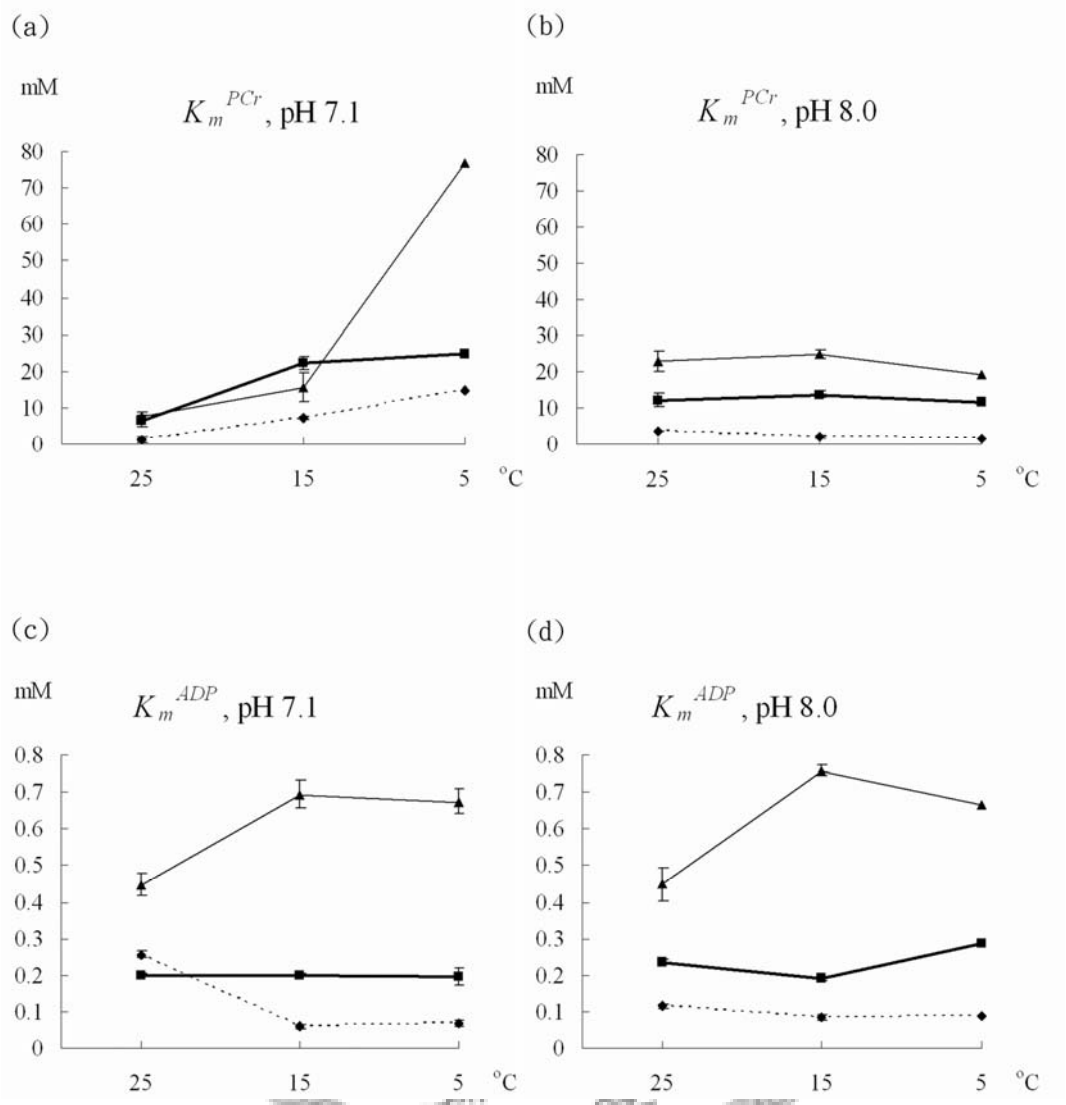


Figure 4. K_m of RM-, M1-, and M3-CK at different temperatures and pHs. K_m values were derived from double-reciprocal plots of the Michaelis-Menten equation. At least 3 replicates from three batches of recombinant protein preparation were conducted in each condition. The blank reaction without enzyme was subtracted from the absorption values. K_m^{PCr} at (a) pH 7.1, (b) pH 8.0; K_m^{ADP} at (c) pH 7.1, (d) pH 8.0. ♦: RM-CK; ■: M1-CK; ▲: M3-CK. Error bars designate standard deviations.

Figure 5.

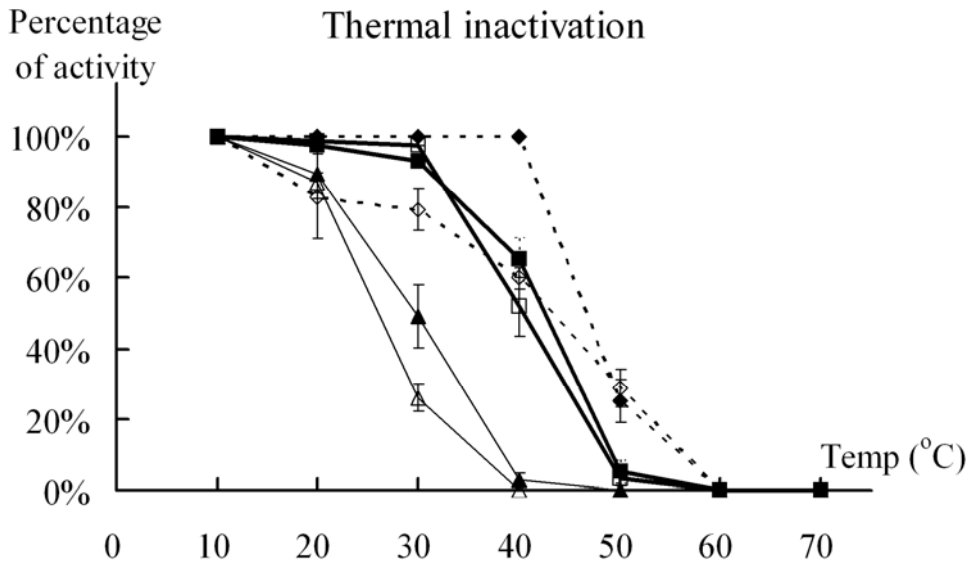


Figure 5. Thermal inactivation curve of RM-, M1-, and M3-CK. Thermal inactivation assays of the three M-CKs are as described in the text, and at least 3 replicates were conducted for each assay. ♦: RM-CK; ■: M1-CK; ▲:M3-CK. Error bars designate standard deviations.

Figure 6.

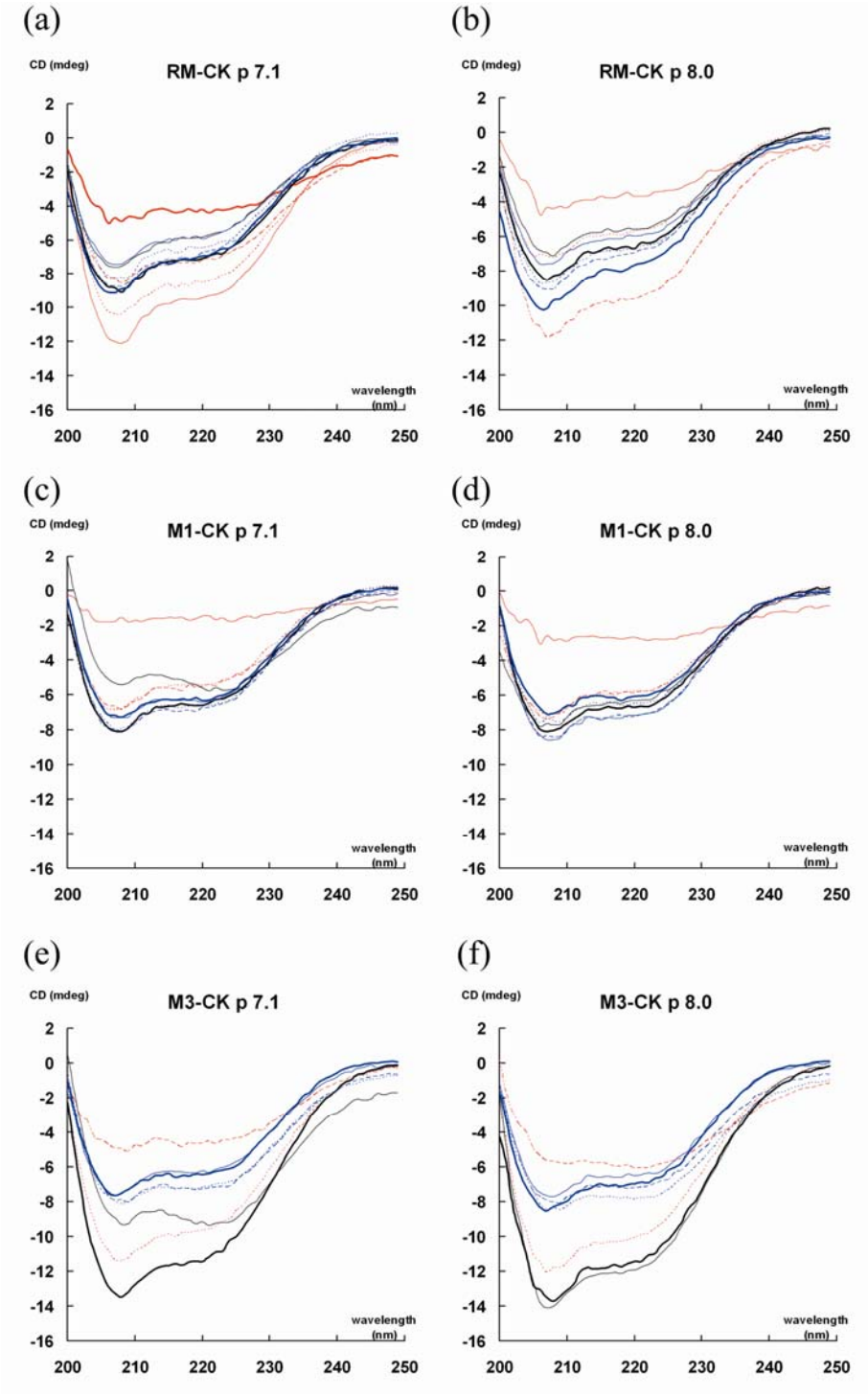


Figure 6. Spectra of far-UV CD spectroscopy of RM-, M1-, and M3-CK at different pHs and temperatures. Far-UV CD spectra were collected as described in the text. Colour line representation of each temperature is presented below. (a)RM-CK in pH 7.1 buffer;

(b)RM-CK in pH 8.0 buffer; (c) M1-CK in pH 7.1 buffer; (d)M1-CK in pH 8.0 buffer; (e)M3-CK in pH 7.1 buffer; (f)M3-CK in pH 8.0 buffer.

: 50 ˚C; : 45 ˚C; : 40 ˚C; : 35 ˚C; : 30 ˚C;

: 25 ˚C; : 20 ˚C; : 15 ˚C; : 10 ˚C; : 5 ˚C.

Figure 7.

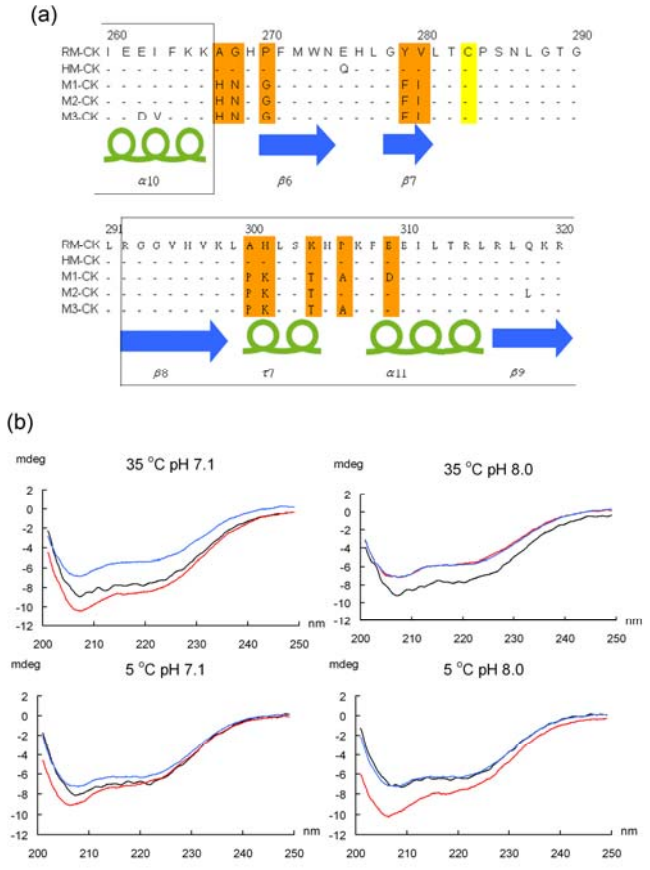


Figure 7. The peptide sequence alignment of RM-CK and carp M1-CK nearby the active site and the CD spectra of RM-CK and RM-CK G268N. The black box is the key residue of active site cysteine 283. The closed boxes and gray boxes are the different residues between two peptides where the gray boxes are the mutation site of this study. The α-helix (curve) and β-sheet (arrow) regions are represented below the sequences. The black frame regions are included in the symmetric structure of the substrate binding site. The mutated residues A267H, G268N and P270G are located outside the symmetric region and secondary structures.

Figure 8.

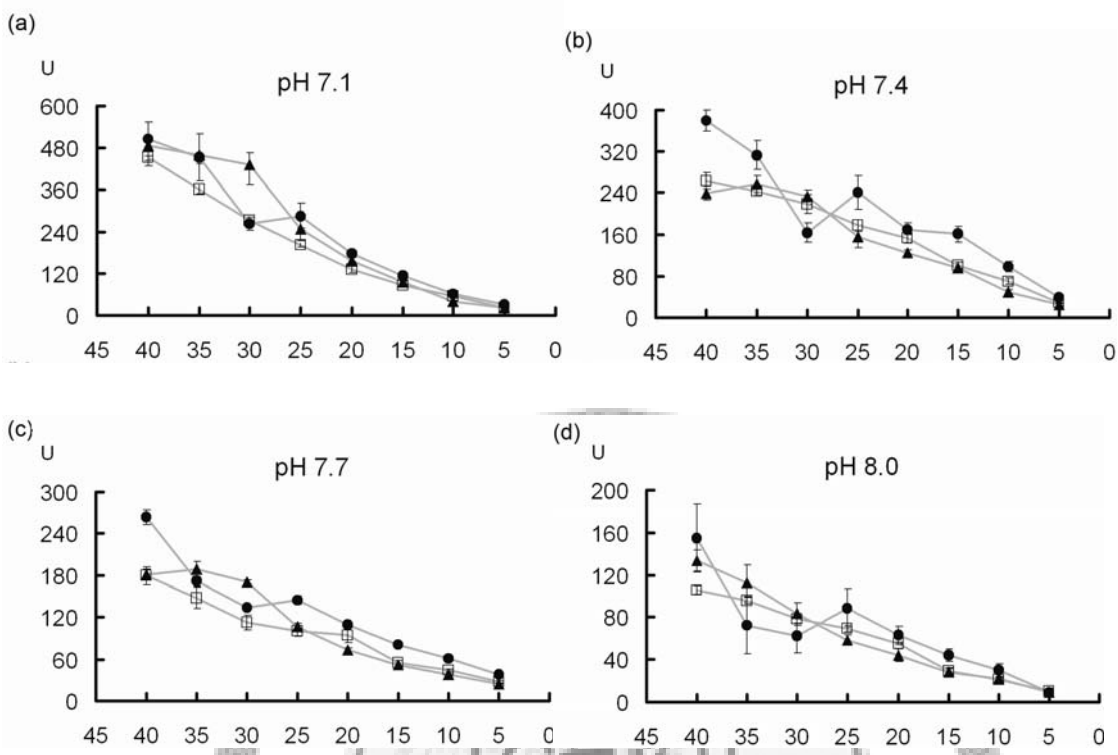


Figure 8. The specific activities of RM-CK mutants. The specific activities for different enzymes in different temperatures and pHs. (a) pH 7. 1; (b) pH 7.4; (c) pH 7.7; (d) pH 8.0. ●: RM-CK A267H; ▲: RM-CK P270G; □: RM-CK A329S. The 1 U is 1 μmole of creatine formed per minute per milligram of enzyme. The horizontal bars are standard division (SD).

Figure 9.

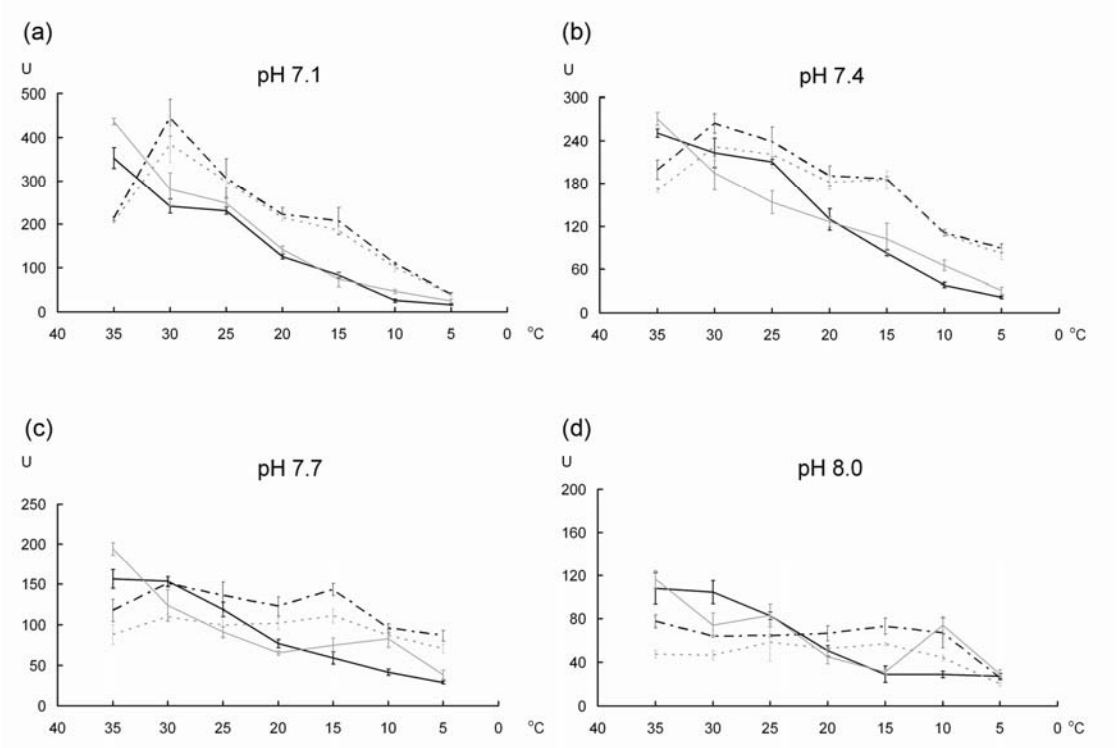


Figure 9. Specific activities and thermal inactivation curves of RM-CK, RM-CK G268N, M1-CK and M1-CK N268G at different pH and temperature. Specific activities of the two wild-type and mutant M-CKs at (a) pH 7. 1, (b) pH 7.4, (c) pH 7.7, (d) pH 8.0, and at temperature from 35 to 5 °C. Thermal inactivation curves at (e) pH 7. 1 and (f) pH 8.0. ▬▬ : RM-CK; ▬▬ : RM-CK G268N; ▬ ▬ : M1-CK and ▪▪▪▪▪▪▪▪▪▪▪▪ : M1-CK N268G. 1 U is 1 μmole of creatine formed per minute per milligram of enzyme. Vertical bars are standard divisions (SD). Thermal inactivation curves are normalized with the highest specific activity of enzymes of 10 °C heat treatment, (n>3, $P < 0.05$).

Figure 10.

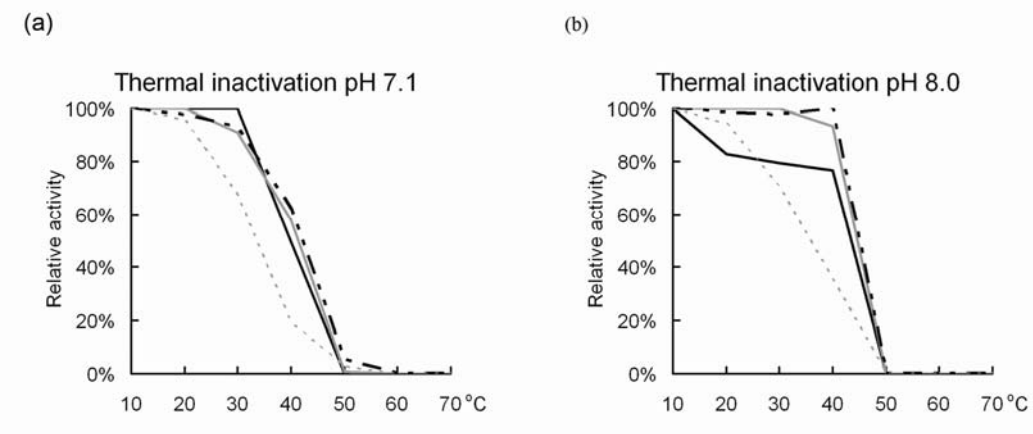


Figure 10. Thermal inactivation curves of RM-CK, RM-CK G268N, M1-CK and M1-CK N268G at different pH. Thermal inactivation curves at (e) pH 7. 1 and (f) pH 8.0. ▬▬ : RM-CK; ▬ ▬ : RM-CK G268N; ▬ ▪ ▬ : M1-CK and ..... : M1-CK N268G. Thermal inactivation curves are normalized with the highest specific activity of enzymes of 10 °C heat treatment.

Figure 11.

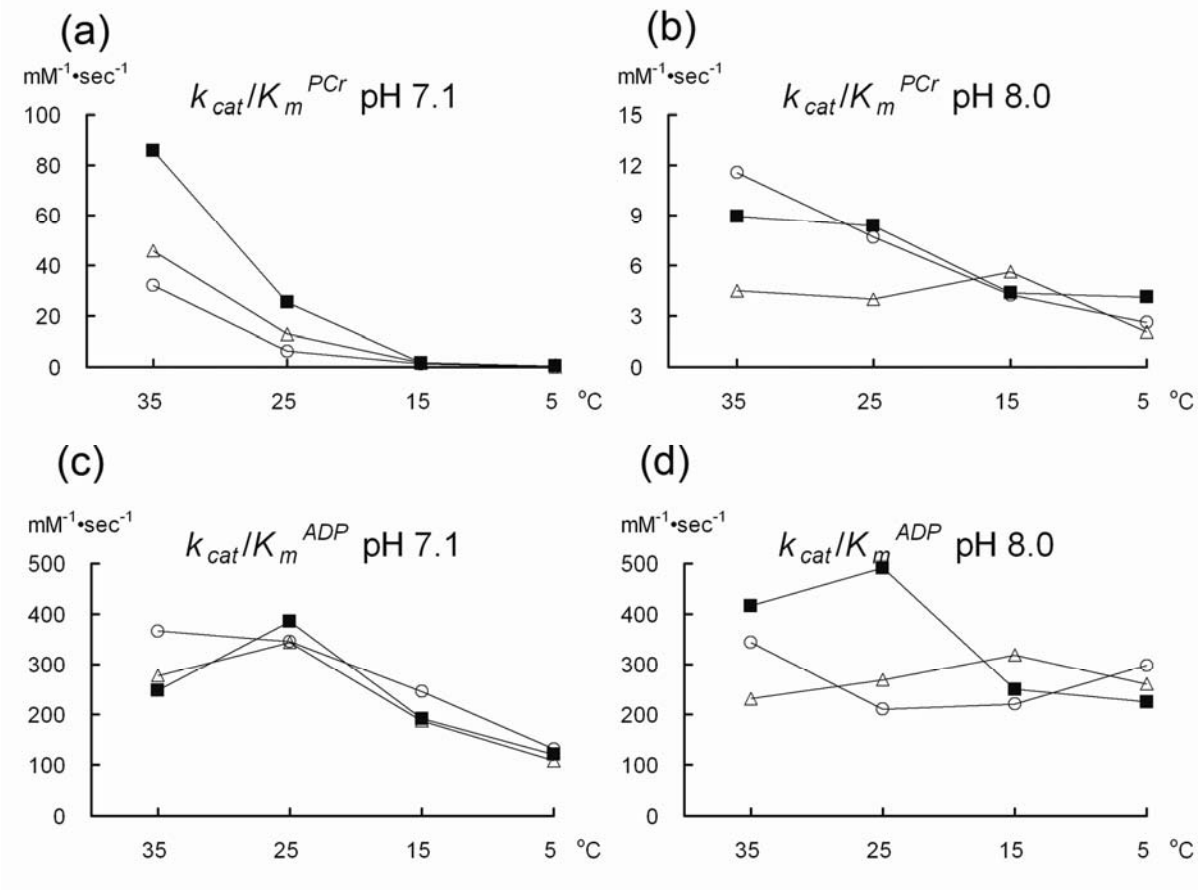


Figure 11. The k_{cat}/K_m^{Pcr} and k_{cat}/K_m^{ADP} of RM-CK mutants. The thermodynamic and kinetic data derivated catalysis ability of different RM-CK mutants in different temperatures and pHs. (a) k_{cat} / K_m^{PCr} in pH 7. 1; (b) k_{cat} / K_m^{PCr} in pH 8.0; (c) k_{cat} / K_m^{ADP} in pH 7. 1; (d) k_{cat} / K_m^{ADP} in pH 8.0. ○: RM-CK A267H; △: RM-CK P270G; ■: RM-CK A329S, (n>3, $p < 0.05$).

Figure 12.

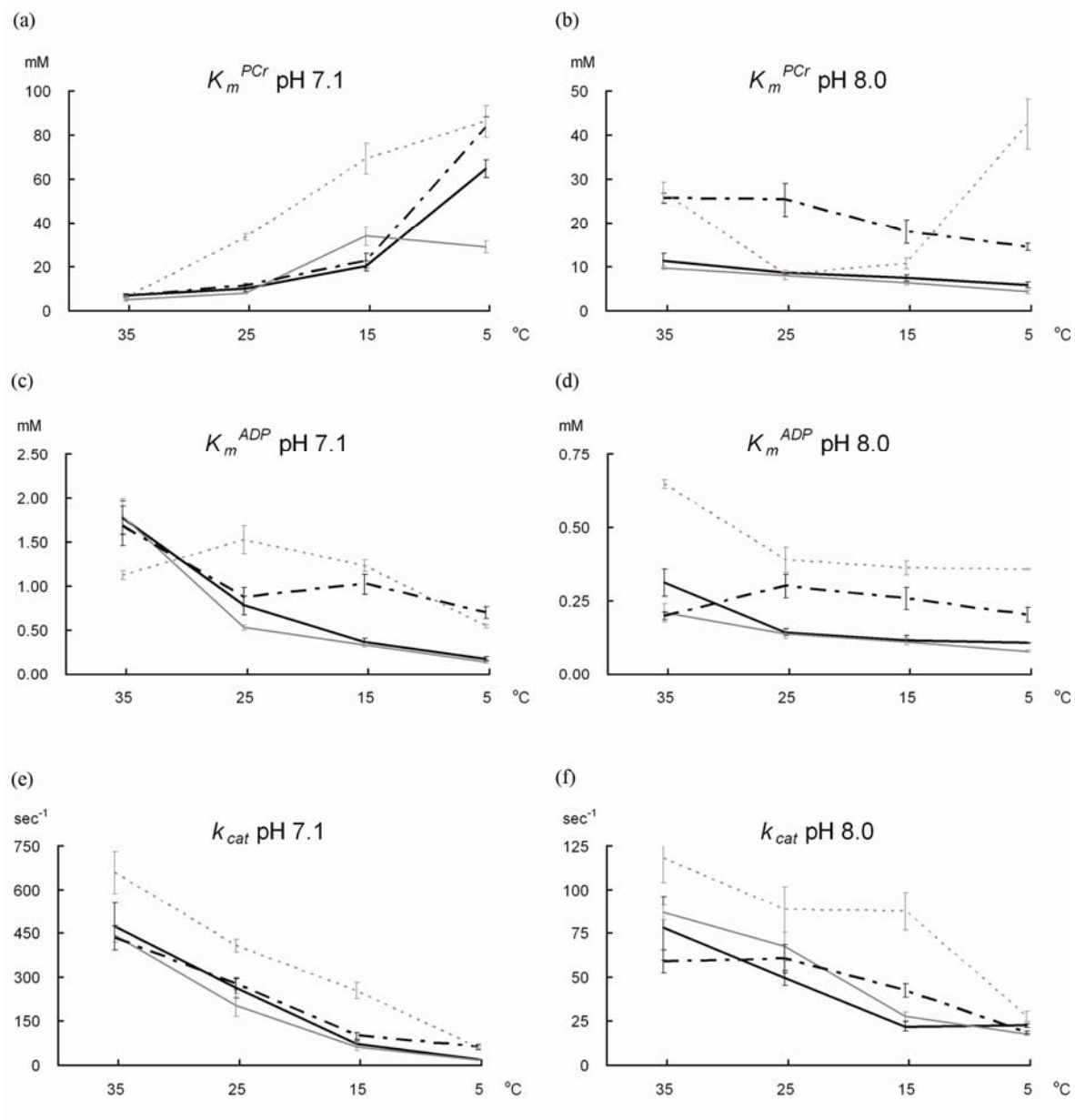


Figure 12. Kinetic analyses of RM-CK, RM-CK G268N, M1-CK and M1-CK N268G at different pH and temperature. (a) K_m^{PCr}, at pH 7.1; (b) K_m^{PCr}, at pH 8.0; (c) K_m^{ADP}, at pH 7.1; (d) K_m^{ADP}, at pH 8.0. (e) k_{cat}, at pH 7.1; (f) k_{cat}, at pH 8.0 of RM-CK, M1-CK and their mutants. ▬▬ : RM-CK; ▬▬ : RM-CK G268N; ▬ ▬ : M1-CK and ····· : M1-CK N268G, ($n>3$, $p < 0.05$).

Figure 13.

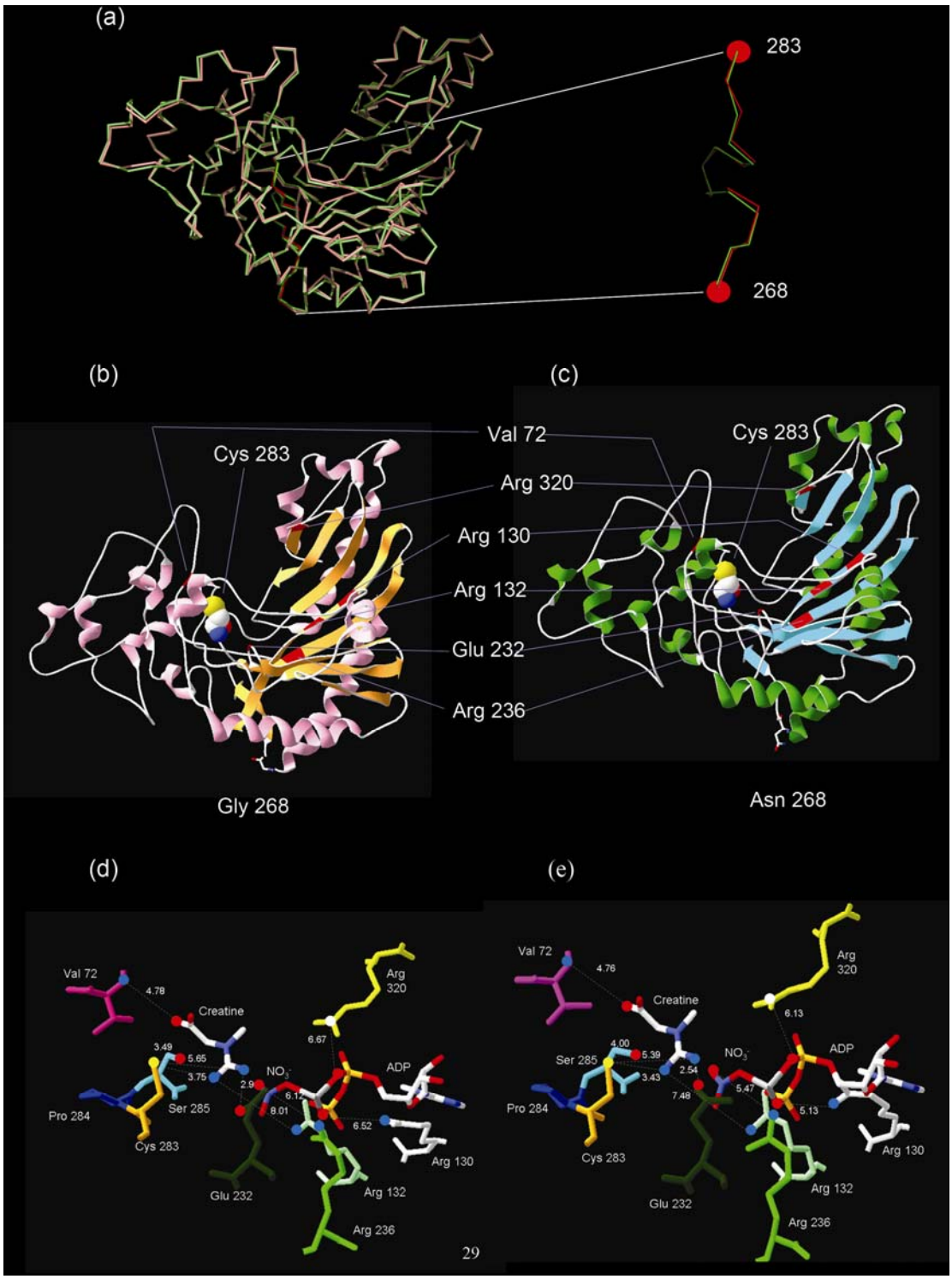


Figure 13. Crystal structures of RM-CK (2crk) and RM-CK G268N and fine structures of the active sites. (a) The superimposed backbone structures of RM-CK (red) and RM-CK G268N (green). The backbone structures of the two molecules almost fully overlapped with

the same conformations. (b) The ribbon models of RM-CK and (c) RM-CK G268N. The cysteine283 are marked as CPK model. The selected residues for calculating the distances between them and the cysteine 283 are marked with red and labeled. These models show the positions of the selected residues in their whole protein molecules. The distances between the selected residues of (d) RM-CK and (e) RM-CK G268N to the substrate analog complexes are shown. The residues which would form hydrogen bonds to adapt the intermediate substrate analogs are labeled. The distances of valine 72 N-Cr O; arginine 130 Nη1-ADP O2β; arginine 132 Nη1–Cr Nβ; glutamic acid 232 Oε1–Cr Nα; arginine 236 Nη2–Nitrate O2; cysteine 283 S–Cr Nα; and arginine 320 Nη1-ADP O1α are presented in Å. Distances between the selected residues are shorter in RM-CK G268N. All the models were processed using the Swiss-model PDB viewer 4.01.

Figure 14.

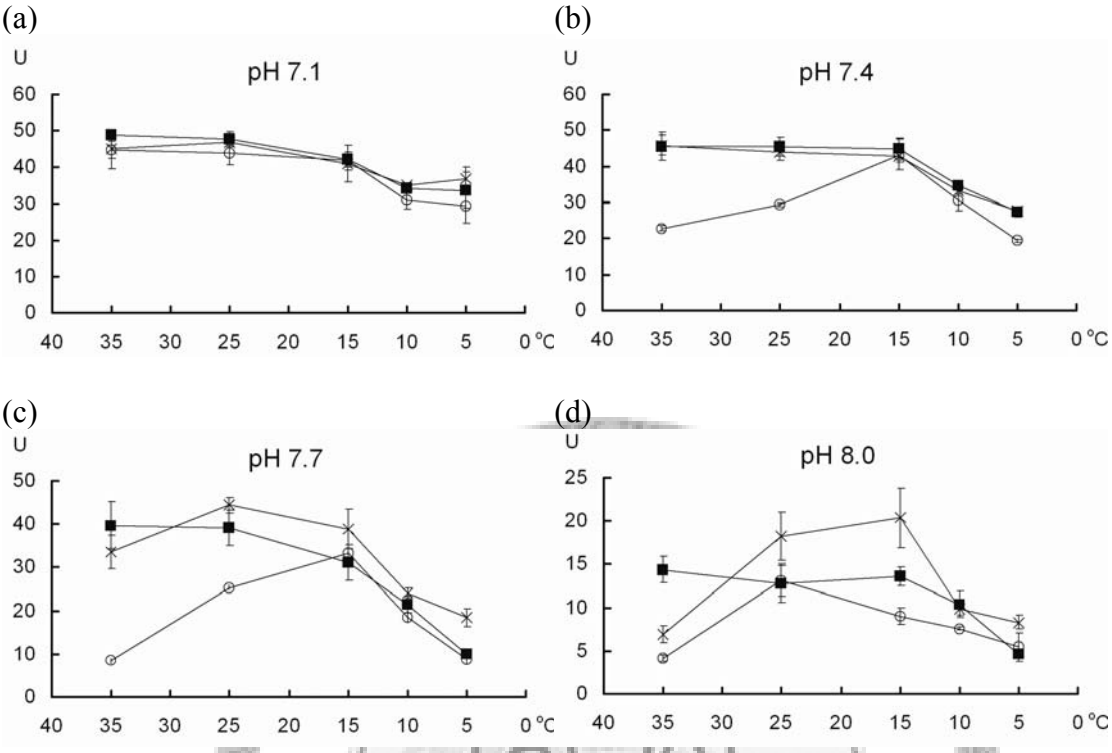


Figure 14. Specific activities RM-CK, M1-CK and RM-CK G268N with dADP substituting ADP as substrate. Specific activities with dADP substitution at different temperature and pH. (a) pH 7.1; (b) pH 7.4; (c) pH 7.7; (d) pH 8.0. —o— : RM-CK; —■— : RM-CK G268N; —✕— : carp M1-CK. 1 U is 1 μmole of creatine formed per minute per milligram of enzyme. Vertical bars are standard divisions (SD), ($n>3$, $P < 0.05$).

Figure 15.

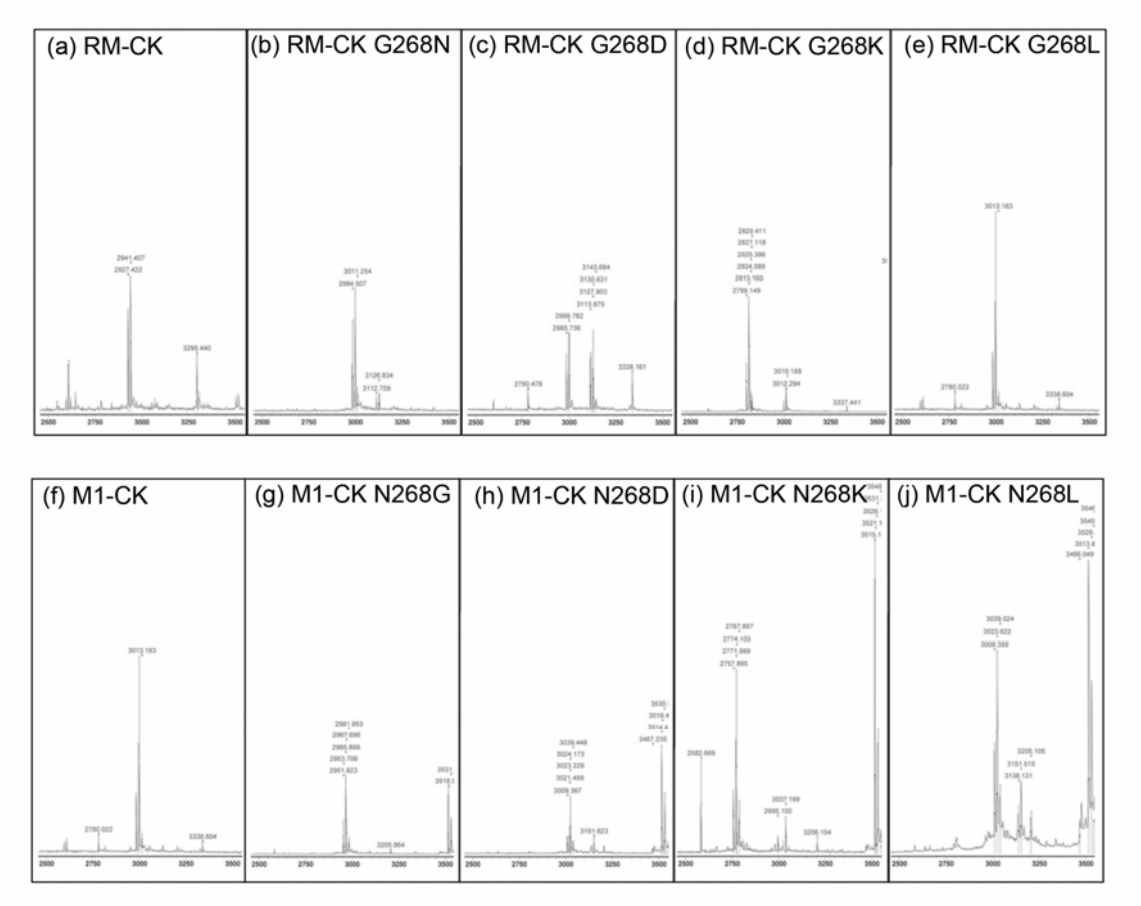


Figure 15. The mass spectra of RM-CK and M1-CK mutants of residue 268. The MALDI-TOF spectra m/z between 2500 to 3500. (a)RM-CK; (b)RM-CK G268N; (c)RM-CK G268D; (d)RM-CK G268K; (e)RM-CK G268L; (f)M1-CK; (g) M1-CK N268G; (h) M1-CK N268D; (i) M1-CK N268K; (j) M1-CK N268L.

Figure 16.

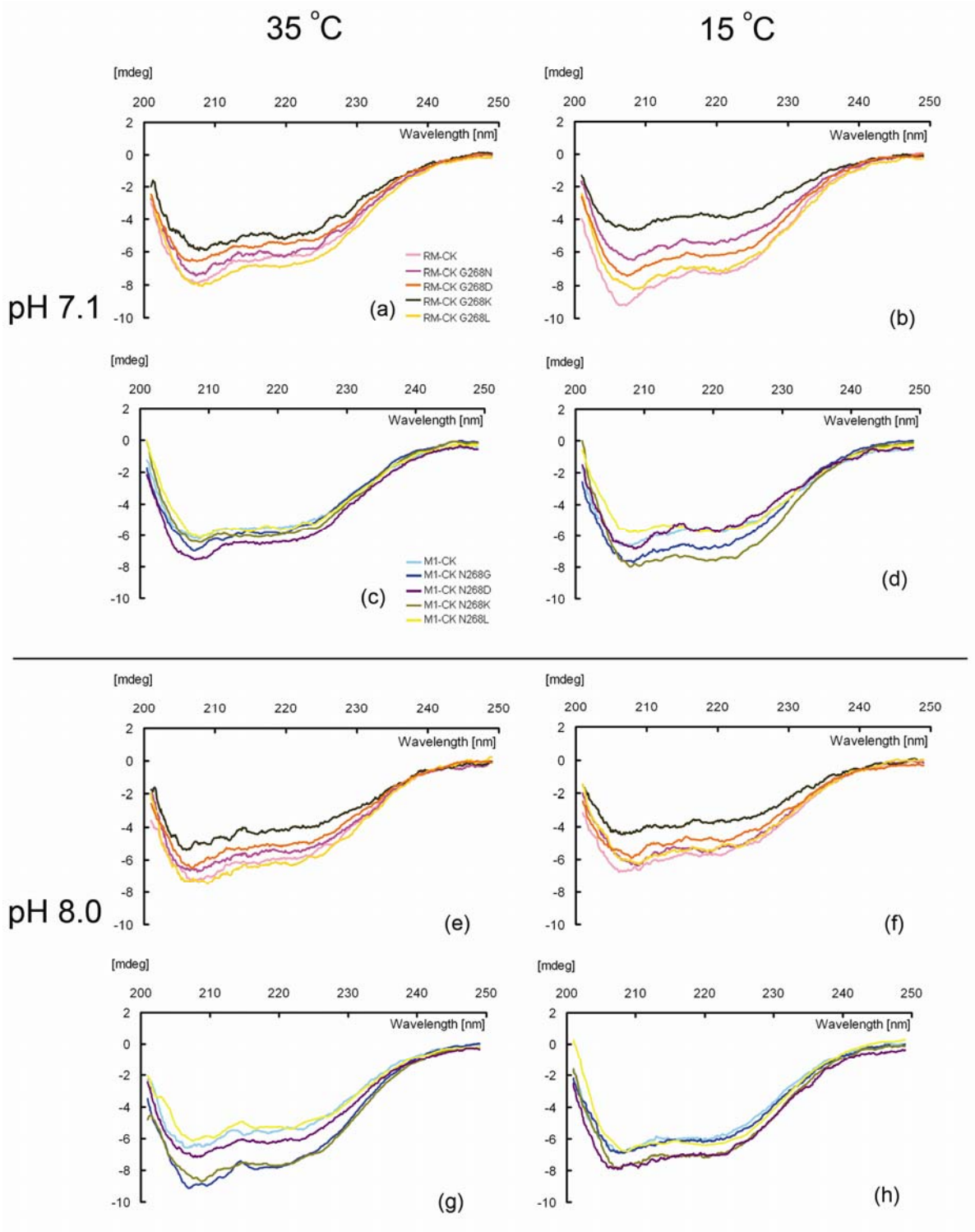


Figure 16. The CD spectra of RM-CK, M1-CK and mutants at different temperature and pH. (A)RM-CK and mutants at 35 °C, pH 7.1. (B)RM-CK and mutants at 15 °C, pH 7.1.

(C)M1-CK and mutants at 35 °C, pH 7.1. (D) M1-CK and mutants at 15 °C, pH 7.1. (E)RM-CK and mutants at 35 °C, pH 8.0. (F)RM-CK and mutants at 15 °C, pH 8.0. (G) M1-CK and mutants at 35 °C, pH 8.0. (H) M1-CK and mutants at 15 °C, pH 8.0. —: RM-CK; — : RM-CK G268N; —: RM-CK G268D; —: RM-CK G268K; —: RM-CK G268L; —: M1-CK; —: M1-CK N268G; —: M1-CK N268D; —: M1-CK N268K; —: M1-CK N268L.

Figure 17.

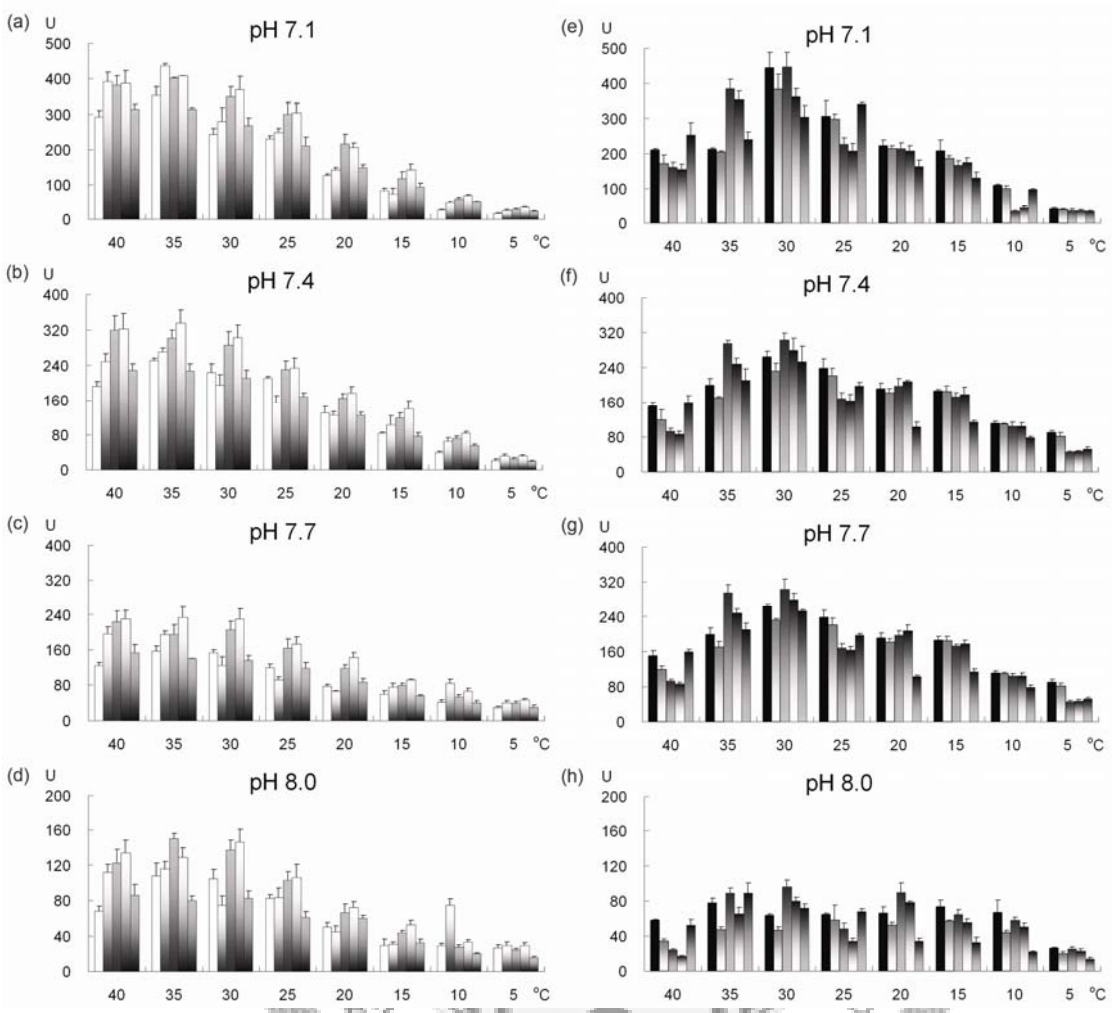


Figure 17. The specific activity of RM-CK, M1-CK and mutants. (A) RM-CK and mutants at pH 7.1. (B) RM-CK and mutants at pH 7.4. (C) RM-CK and mutants at pH 7.7. (D) RM-CK and mutants at pH 8.0. (E) M1-CK and mutants at pH 7.1. (F) M1-CK and mutants at pH 7.4. (G) M1-CK and mutants at pH 7.7. (H) M1-CK and mutants at pH 8.0. : RM-CK; : RM-CK G268N; : RM-CK G268D; : RM-CK G268K; : RM-CK G268L; : M1-CK; : M1-CK N268G; : M1-CK N268D; : M1-CK N268K; : M1-CK N268L.

Figure 18.

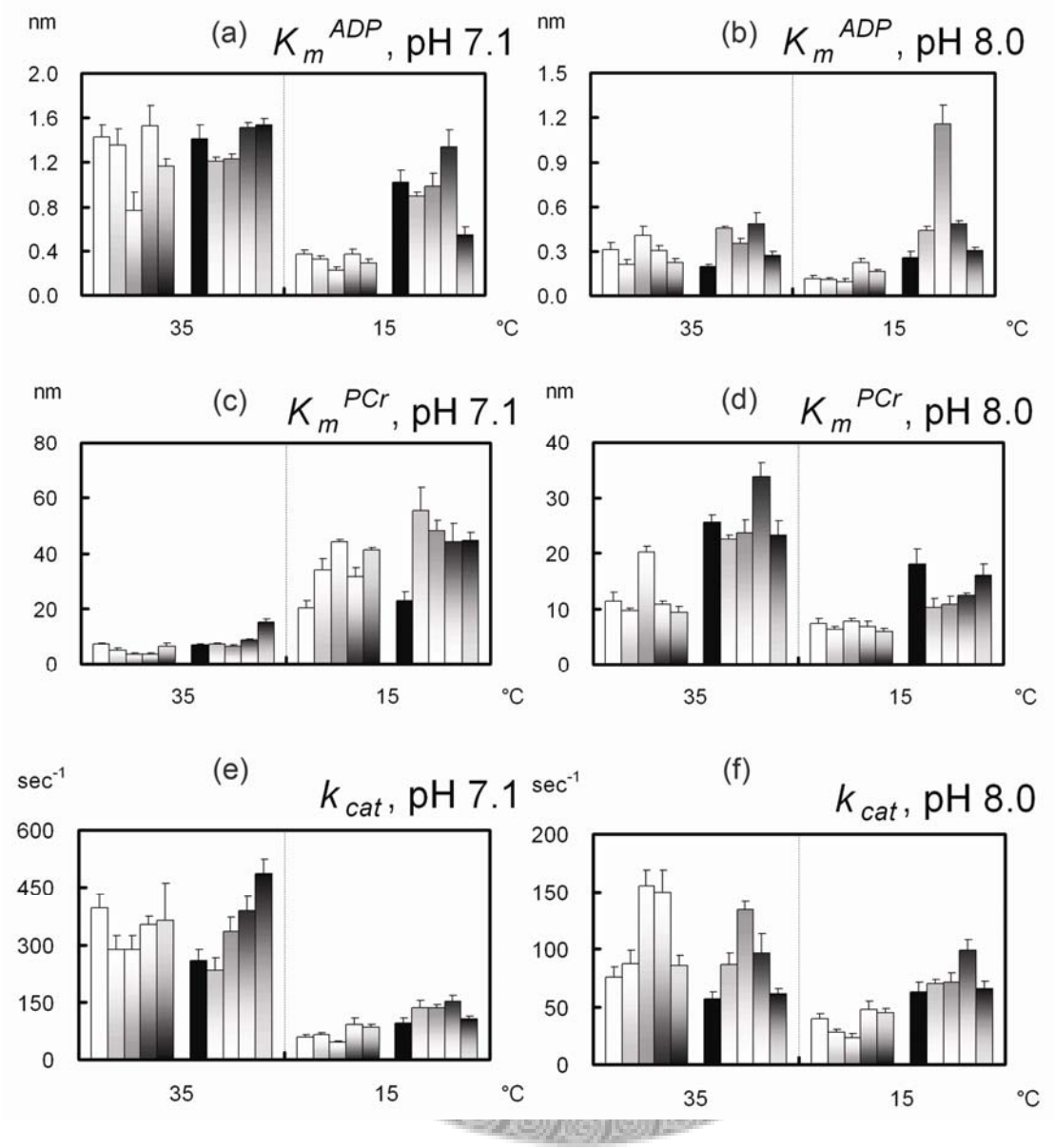


Figure 18. The kinetic data of RM-CK, M1-CK and mutants. (A) K_m^{ADP} at pH 7.1. (B) K_m^{ADP} at pH 8.0. (C) K_m^{PCr} at pH 7.1. (D) K_m^{PCr} at pH 8.0. (E) k_{cat} at pH 7.1. (F) k_{cat} at pH 8.0. : RM-CK; : RM-CK G268N; : RM-CK G268D; : RM-CK G268K; : RM-CK G268L; : M1-CK; : M1-CK N268G; : M1-CK N268D; : M1-CK N268K; : M1-CK N268L.

Figure 19.

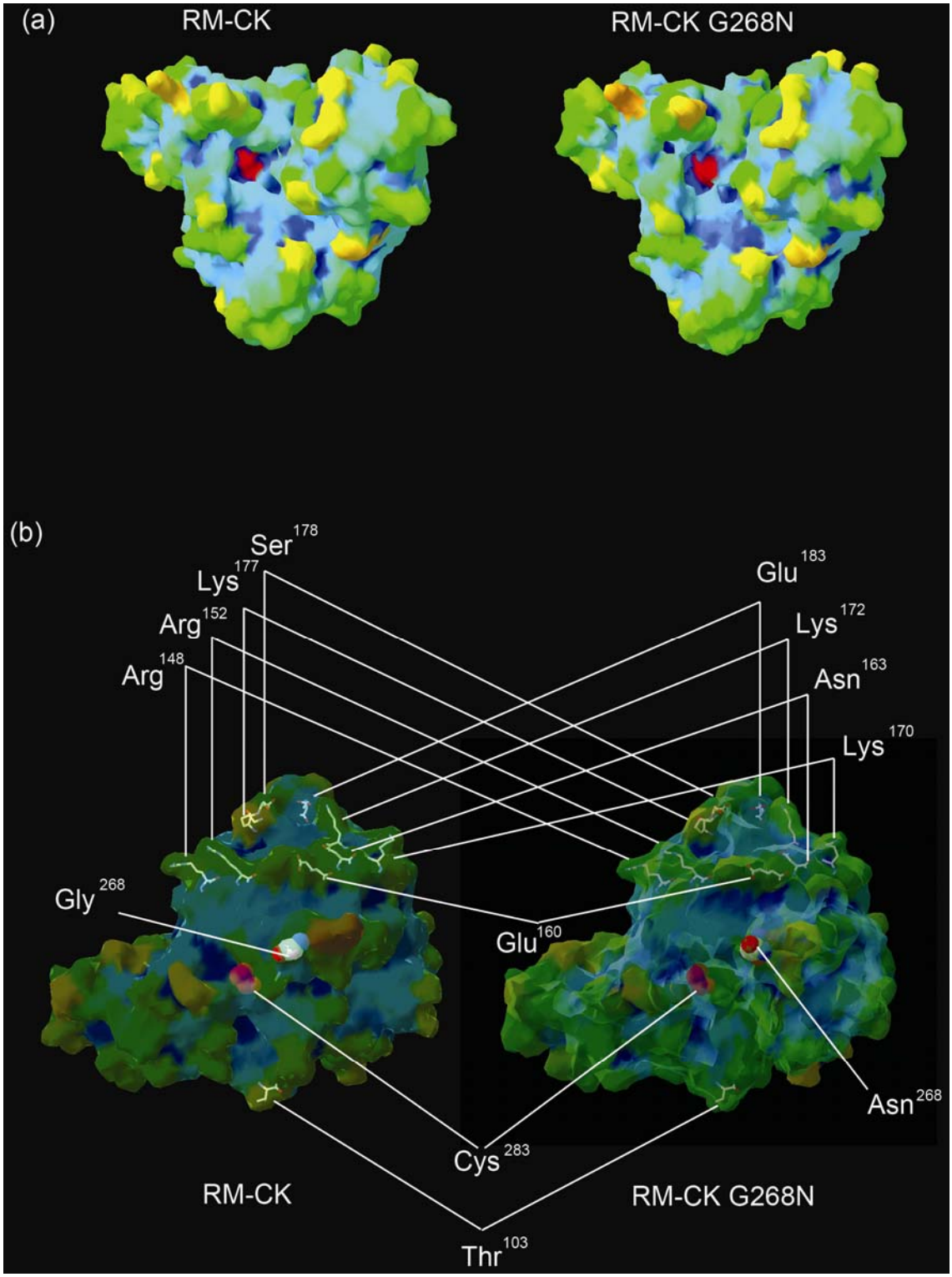


Figure 19. The solvent accessible surface of RM-CK and RM-CK G268N. (A) The molecule surface of RM-CK and RM-CK G268N which yellow to blue indicated the

percentage of solvent accessible surface of each residue. Yellow was highly accessible and blue was low accessible residues. Red indicated the residue 268 (glycine 268 or asparagine 268). (B) The selected residues located around the residue 268 and their solvent accessible surface. Orange atoms indicated the cysteine 283, colored with CPK atoms indicate the glycine 268 of RM-CK or asparagine 268 of RM-CK G268N.

Figure 20.

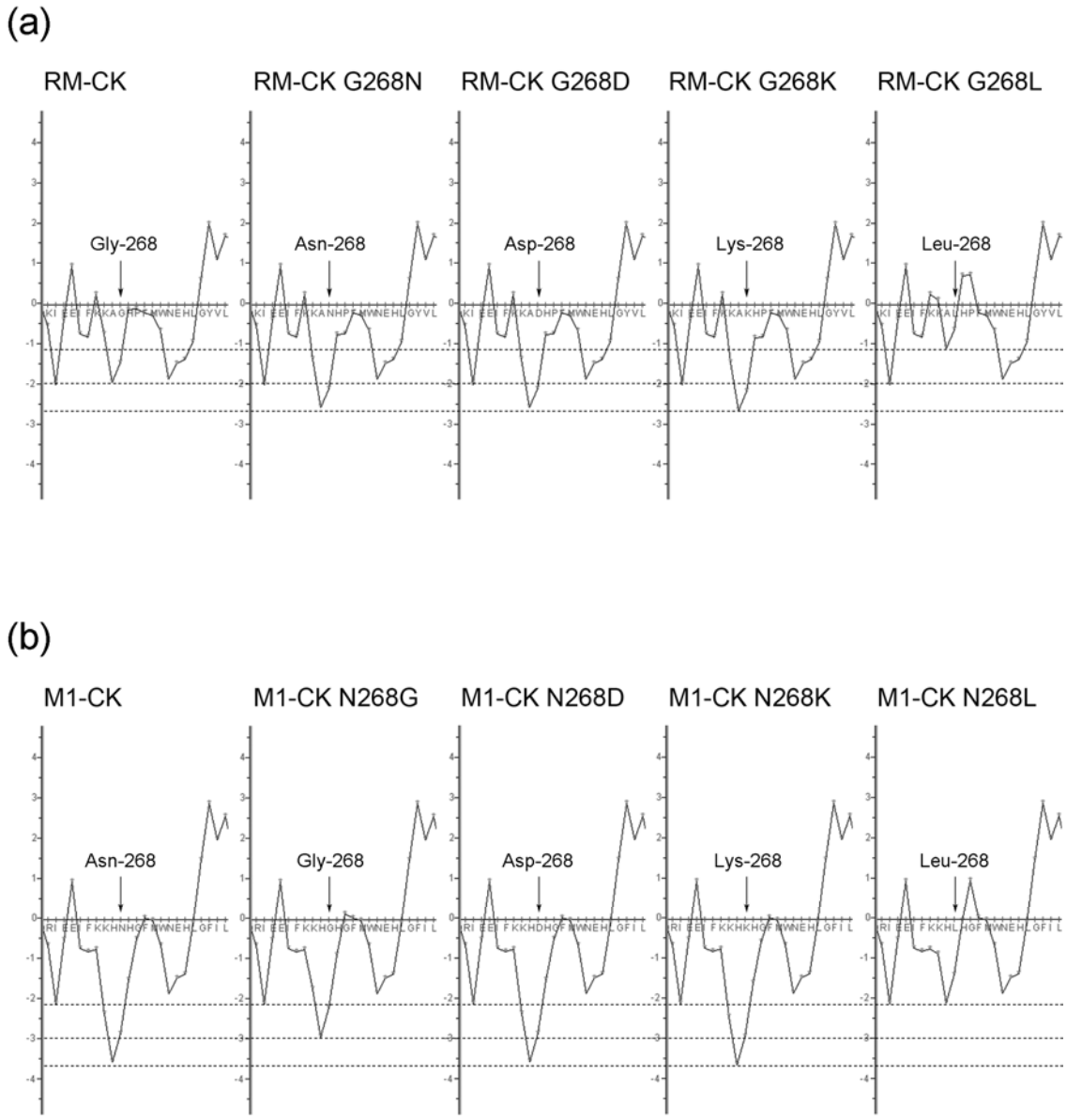


Figure 20. The simulated hydrophobicity of different M-CKs. (a) RM-CK and mutants; (b) M1-CK and mutants. y axis: hydrophobicity index; x axis: amino acid sequence from 259 to 281.

Figure 21.

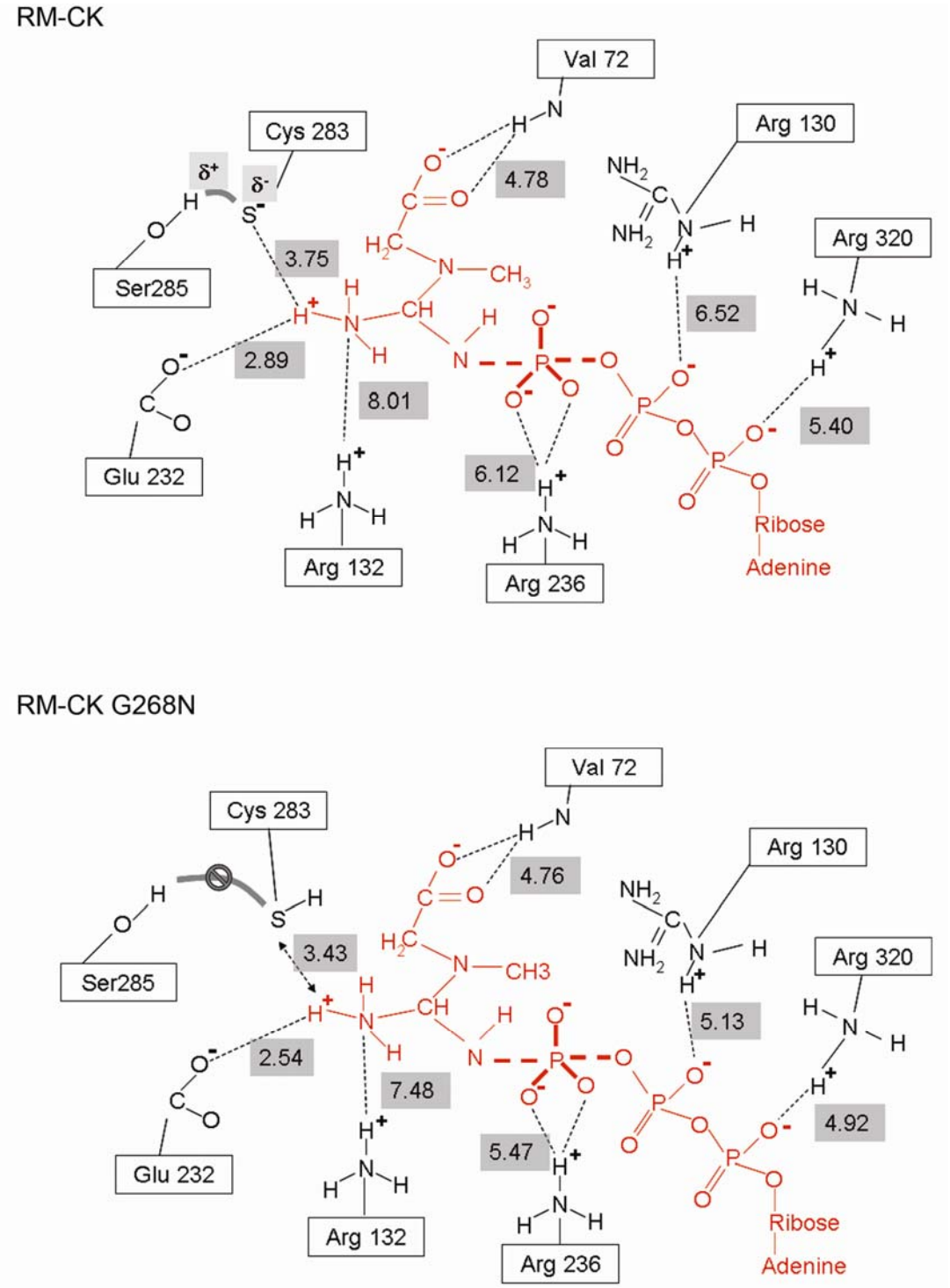


Figure 21. Cartoons of RM-CK and RM-CK G268N active sites at low temperature and pH above 7.7. Gray boxes indicate calculated distances of atoms (unit: Å).

Figure 22.

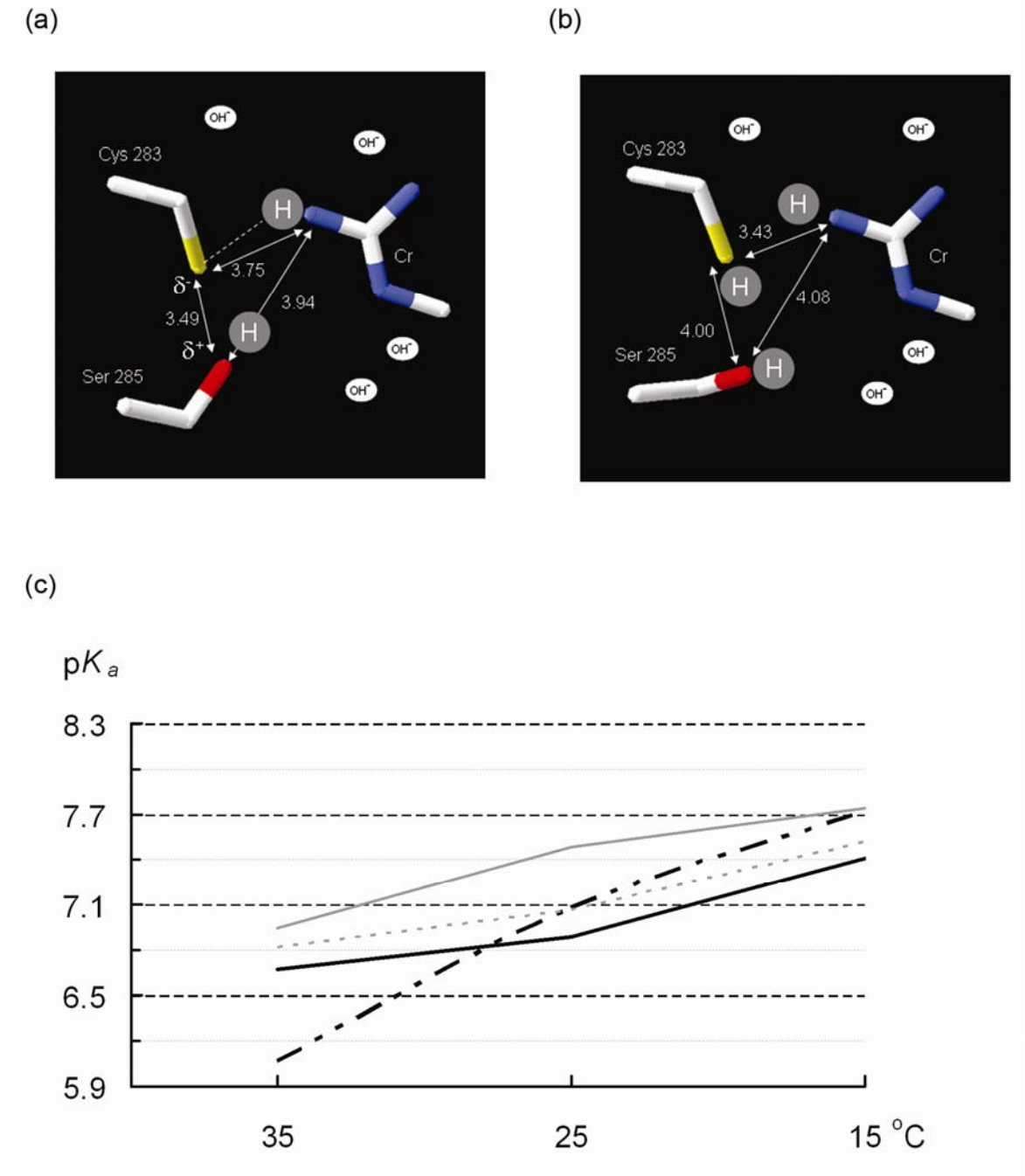


Figure 22. The atom positions of cysteine 283, serine 285 and Cr amine group of RM-CK and RM-CK G268N, and *pKa* values of RM-CK, M1-CK and their mutants derived from kinetic results. Distances of atom positions of the thiol of cysteine 283, hydroxyl of serine 285 and the amine group of Cr are shown in (a) RM-CK (2crk) and (b) RM-CK G268N.

At pH 7.7 or above the thiol group of cysteine 283 in RM-CK is presented to be deprotonated, while that of RM-CK G268N is protonated. Red atom is oxygen; blue is nitrogen and yellow is sulfur. Distances between atoms are presented in Å. These models were processed by the Swiss-model PDB viewer 4.01. (c) *pKa* of RM-CK, M1-CK and mutants. ▬▬ : RM-CK; ▬▬ : RM-CK G268N; ▬ ▪ ▬ : M1-CK and ▪▪▪▪▪▪▪▪▪▪▪▪ : M1-CK N268G.

TABLES

Table 1. The primer pairs of site directed mutagenesis of RM-CK and M1-CK residue 268.

	Internal primer pair
RM-CK G268N	5′-CACATGAAGGGGTGGTTAGCTTTC-3′
	5′-CTTTAAGAAAGCTAACCACCCC-3′
RM-CK G268D	5′-TTAAGAAAGCTGACCACCCCTTCATG-3′
	5′-CATGAAGGGGTGGTCAGCTTTCTTAA-3′
RM-CK G268K	5′-TTAAGAAAGCTAAGCACCCCTTCATG-3′
	5′-CATGAAGGGGTGCTTAGCTTTCTTAA-3′
RM-CK G268L	5′-TTAAGAAAGCTCTCCACCCCTTCATG-3′
	5′-CATGAAGGGGTGGAGAGCTTTCTTAA-3′
	Internal primer pair
M1-CK N268G	5′-TTCAAGAAGCACGGCCATGGATTCATG-3′
	5′-CATGAATCCATGGCCGTGCTTCTTGAA-3′
M1-CK N268D	5′-GAAGCACGACCATGGATTCATGTGG-3′
	5′-GAATCCATGGTCGTGCTTCTTGAAA-3′
M1-CK N268K	5′-GAAGCACAAGCATGGATTCATGTGG-3′
	5′-GAATCCATGCTTGTGCTTCTTGAAA-3′
M1-CK N268L	5′-GAAGCACCTCCATGGATTCATGTGG-3′
	5′-GAATCCATGGAGGTGCTTCTTGAAA-3′

Table 2. Biochemical kinetic values of RM-, M1-, and M3-CK at different temperatures and pHs. k_{cat} values were derived using the Michaelis-Menten equation. k_{cat}/K_m^{PCr} and k_{cat}/K_m^{ADP} were calculated using the experimental data in Figure 3.

	Assay	Temp. (°C)	k_{cat} (x 10^3 sec^{-1})	k_{cat}/K_m^{PCr} (x 10^3 $mM^{-1}\cdot sec^{-1}$)	k_{cat}/K_m^{ADP} (x 10^3 $mM^{-1}\cdot sec^{-1}$)	*Ea* (kJ · $mole^{-1}$)
pH 7.1						
		25	8.31 ± 0.47	6.66	32.39	
	RM-CK	15	4.60 ± 0.65	0.64	79.36	63.91
		5	1.31 ± 0.16	0.09	19.68	
		25	17.82 ± 2.09	2.82	89.94	
	M1-CK	15	12.85 ± 0.88	0.58	64.87	38.34
		5	5.88 ± 0.55	0.24	30.17	
		25	35.60 ± 1.17	4.62	79.51	
	M3-CK	15	13.61 ± 1.16	0.87	19.61	79.81
		5	3.52 ± 0.72	0.05	5.22	
pH 8.0						
		25	4.66 ± 0.93	1.25	40.82	
	RM-CK	15	1.80 ± 0.16	0.93	21.93	64.41
		5	0.72 ± 0.12	0.42	8.33	
		25	7.75 ± 0.50	0.64	32.79	
	M1-CK	15	6.00 ± 0.47	0.43	31.60	28.36
		5	3.41 ± 0.34	0.30	11.97	
		25	12.42 ± 0.56	0.54	27.73	
	M3-CK	15	3.97 ± 0.33	0.16	5.25	45.79
		5	3.25 ± 0.36	0.17	4.88	

Table 3. The kinetic data of RM-CK, RM-CK mutants and carp M1-CK show the similar pattern in different pHs.

	pH 7.1				pH 8.0			
Temperature (°C)	35.0	25.0	15.0	5.0	35.0	25.0	15.0	5.0
K_m^{PCr}								
RM-CK	7.1 ± 0.4	10.1 ± 1.4	20.4 ± 2.3	64.8 ± 3.9	11.3 ± 1.6	8.7 ± 0.5	7.4 ± 0.8	5.9 ± 0.7
RM-CK A267H	20.6 ± 1.6	52.9 ± 2.0	81.5 ± 5.4	111.7 ± 14.9	11.8 ± 1.0	9.3 ± 0.5	7.8 ± 0.5	6.9 ± 0.2
RM-CK G268N	5.2 ± 0.8	8.1 ± 0.4	33.9 ± 4.1	28.9 ± 2.8	9.7 ± 0.3	8.0 ± 1.1	6.3 ± 0.5	4.3 ± 0.4
RM-CK P270G	9.0 ± 0.9	23.4 ± 1.2	54.6 ± 5.4	114.6 ± 13.1	15.3 ± 1.6	15.3 ± 0.7	8.8 ± 1.4	7.7 ± 1.2
M1-CK	6.7 ± 0.6	11.3 ± 1.0	22.8 ± 3.3	83.6 ± 4.6	25.7 ± 1.2	25.3 ± 3.7	18.0 ± 2.7	14.6 ± 0.9
K_m^{ADP}								
RM-CK	1.8 ± 0.2	0.8 ± 0.1	0.4 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
RM-CK A267H	1.8 ± 0.2	1.0 ± 0.0	0.4 ± 0.0	0.1 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
RM-CK G268N	1.8 ± 0.2	0.5 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
RM-CK P270G	1.5 ± 0.1	0.9 ± 0.0	0.5 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
M1-CK	1.7 ± 0.2	0.9 ± 0.1	1.0 ± 0.1	0.7 ± 0.1	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0
k_{cat}								
RM-CK	473.3 ± 31.9	263.7 ± 15.9	72.4 ± 9.5	20.1 ± 2.5	78.0 ± 11.5	49.6 ± 7.4	22.0 ± 3.0	22.6 ± 3.1
RM-CK A267H	658.4 ± 80.4	331.9 ± 34.0	96.6 ± 10.5	19.3 ± 1.1	136.4 ± 18.2	71.3 ± 4.2	33.2 ± 2.8	18.0 ± 1.0
RM-CK G268N	443.2 ± 44.6	204.1 ± 9.7	63.3 ± 5.9	16.8 ± 2.5	87.0 ± 12.5	67.4 ± 5.6	27.8 ± 2.5	17.6 ± 1.7
RM-CK P270G	413.2 ± 26.6	301.1 ± 38.8	88.7 ± 12.4	18.0 ± 1.6	68.7 ± 4.2	61.2 ± 8.1	49.6 ± 2.5	15.6 ± 0.6
M1-CK	433.7 ± 41.8	276.1 ± 19.0	99.6 ± 11.9	62.0 ± 8.1	58.9 ± 6.7	60.3 ± 8.0	42.2 ± 3.7	18.5 ± 1.0
k_{cat}/K_m^{PCr} & k_{cat}/K_m^{ADP}								
RM-CK	66.7 & 266.5	26.1 & 338.1	3.5 & 197.5	0.3 & 115.4	6.9 & 250.3	5.7 & 345.8	3.0 & 189.3	3.8 & 212.3
RM-CK A267H	31.9 & 366.8	6.3 & 346.8	1.2 & 245.2	0.2 & 132.0	11.6 & 344.6	7.7 & 211.3	4.3 & 221.4	2.6 & 298.4
RM-CK G268N	85.9 & 247.3	25.3 & 384.8	1.9 & 191.7	0.6 & 121.1	8.9 & 415.9	8.4 & 492.2	4.4 & 250.0	4.1 & 225.6
RM-CK P270G	45.9 & 278.0	12.9 & 343.7	1.6 & 187.8	0.2 & 107.5	4.5 & 230.3	4.0 & 268.0	5.6 & 318.5	2.0 & 259.9
M1-CK	64.3 & 257.2	24.3 & 314.8	4.4 & 97.6	0.7 & 88.8	2.3 & 297.7	2.4 & 201.3	2.3 & 164.2	1.3 & 91.5

The data represented in mean ± standard division (SD). The unit of K_m^{ADP} and K_m^{PCr} is mM. The unit of k_{cat} is 1/sec. The unit of k_{cat}/K_m is 1/(mM·sec).

Table 4. Crystallographic analysis of RM-CK G268N.

Crystallographic data-collection and refinement statistic	
Space group	I4
Unit-Cell parameters (Å)	
a = b	179.53
c	68.35
Date collection	
Wavelength (Å)	1.54
No. of reflections	62110
No. of unique reflections	16186
Average multiplicity	3.8
Completeness (%)	97.4 (92.8)*
〈 I/σ(I)〉	21 (2.8)*
Rmerge (%)	7.6 (51)*
Resolution (Å)	50 - 3.3
Refinement	
Refinement program	REFMACS
Resolution range (Å)	50 – 3.3
Rwork / Rfree (%)	19.0 / 30.0

* Values in parentheses are for data in highest resolution shell.

Table 5. Distances between residues surrounding the active site of RM-CK and RM-CK G268N. (a) Distances between each Cα of residue pairs were calculated using the model of RM-CK (2crk) and RM-CK G268N in the Swiss-PDB viewer. Unit is in Å. Numbers in bold font in the RM-CK G268N represent shorter distances comparing to the same residue pairs of RM-CK (2crk). (b) Differences in distances of selected residues Cα to the thiol group of cysteine 283. Δ is their difference.

(a)

RM-CK Cα distances						
RM-CK G268N Cα distances	Val 72	Arg 132	Glu 232	Arg 236	Arg 292	Arg 341
Val 72		20.10	14.86	19.41	17.16	13.96
Arg 132	20.40		13.39	7.44	5.48	11.33
Glu 232	14.86	13.42		7.84	15.10	16.83
Arg 236	19.69	7.29	8.00		11.48	14.90
Arg 292	17.57	5.38	15.12	11.30		7.43
Arg 341	14.10	11.95	16.99	15.25	8.13	

(b)

	RM-CK (2crk)	RM-CK G268N	Δ
Val 72	5.42	5.65	-0.23
Arg 132	15.99	15.41	**0.57**
Glu 232	9.77	9.66	**0.11**
Arg 236	14.39	13.84	**0.54**
Arg 292	14.23	13.61	**0.62**
Arg 341	12.03	11.61	**0.42**

Table 6. The raw data of specific activity of RM-CK, RM-CK A267H, G268N, P270G and M1-CK.

Temperature (°C)	40	35	30	25	20	15	10	5
pH 7.1								
RM-CK	292.8 ± 18.0	352.8 ± 23.9	241.8 ± 16.5	231.3 ± 7.8	125.7 ± 5.4	83.5 ± 6.6	26.0 ± 3.5	16.9 ± 1.3
RM-CK A267H	486.9 ± 43.6	460.4 ± 24.5	432.3 ± 59.7	248.1 ± 32.5	156.5 ± 4.0	95.3 ± 12.1	41.1 ± 2.5	20.7 ± 1.6
RM-CK G268N	391.9 ± 27.1	436.5 ± 7.1	280.6 ± 38.3	249.1 ± 10.3	142.1 ± 7.5	73.6 ± 15.3	47.0 ± 4.9	25.2 ± 3.9
RM-CK P270G	505.2 ± 49.9	452.4 ± 66.5	263.2 ± 18.4	284.0 ± 37.7	176.9 ± 10.7	114.2 ± 9.3	60.6 ± 5.9	30.9 ± 3.8
M1-CK	110.0 ± 11.7	153.6 ± 20.3	229.6 ± 14.4	220.2 ± 15.6	202.1 ± 19.2	86.2 ± 13.6	83.6 ± 19.8	64.3 ± 25.3
pH 7.4								
RM-CK	192.8 ± 10.2	250.7 ± 5.7	222.9 ± 20.4	210.5 ± 3.6	129.9 ± 15.0	83.0 ± 4.0	38.7 ± 3.4	21.7 ± 2.1
RM-CK A267H	239.8 ± 12.4	256.4 ± 17.5	231.9 ± 13.6	155.5 ± 21.4	125.0 ± 4.9	96.5 ± 9.6	49.7 ± 4.2	25.4 ± 3.2
RM-CK G268N	248.8 ± 17.5	270.4 ± 9.0	194.9 ± 23.8	153.8 ± 15.9	126.3 ± 6.8	102.4 ± 21.7	65.7 ± 7.2	31.1 ± 4.8
RM-CK P270G	379.5 ± 20.5	312.6 ± 27.6	162.5 ± 18.1	241.6 ± 32.8	169.8 ± 11.2	160.9 ± 15.2	98.5 ± 10.2	39.6 ± 4.3
M1-CK	112.5 ± 7.8	111.8 ± 4.4	208.9 ± 5.9	212.0 ± 11.5	200.0 ± 8.9	124.2 ± 34.2	95.9 ± 14.7	76.2 ± 4.7
pH 7.7								
RM-CK	124.4 ± 7.0	156.7 ± 11.6	153.6 ± 6.0	118.9 ± 9.0	76.8 ± 5.2	58.9 ± 7.5	41.6 ± 3.9	28.9 ± 2.0
RM-CK A267H	181.0 ± 10.2	188.8 ± 17.5	170.6 ± 10.7	107.3 ± 4.7	72.4 ± 6.0	52.2 ± 2.5	37.8 ± 0.5	24.9 ± 2.2
RM-CK G268N	195.4 ± 17.4	194.1 ± 7.9	123.8 ± 19.6	91.1 ± 6.2	65.1 ± 2.4	74.8 ± 8.7	82.8 ± 10.6	38.7 ± 5.6
RM-CK P270G	263.7 ± 31.9	171.6 ± 26.4	134.7 ± 16.1	144.3 ± 17.9	109.1 ± 7.8	80.9 ± 6.2	61.5 ± 5.9	38.8 ± 3.8
M1-CK	66.6 ± 8.2	57.8 ± 7.7	142.0 ± 11.3	161.8 ± 12.6	171.3 ± 22.3	150.0 ± 18.6	88.3 ± 13.4	93.8 ± 4.3
pH 8.0								
RM-CK	68.5 ± 5.1	107.8 ± 14.5	104.4 ± 10.6	82.7 ± 3.6	50.6 ± 4.8	29.1 ± 7.5	29.0 ± 3.1	27.1 ± 2.7
RM-CK A267H	133.4 ± 7.2	112.3 ± 10.5	83.2 ± 2.6	58.5 ± 3.5	44.3 ± 3.8	28.4 ± 2.5	22.4 ± 1.8	9.0 ± 0.9
RM-CK G268N	112.0 ± 9.0	116.2 ± 7.7	74.1 ± 11.2	83.3 ± 10.2	45.3 ± 6.4	31.1 ± 2.7	74.1 ± 7.6	29.5 ± 3.6
RM-CK P270G	154.8 ± 10.2	72.0 ± 9.3	62.7 ± 2.8	88.4 ± 6.5	63.3 ± 2.7	44.1 ± 2.7	30.0 ± 3.1	9.1 ± 0.9
M1-CK	28.3 ± 4.1	43.3 ± 2.6	88.5 ± 6.0	110.7 ± 8.3	91.6 ± 20.4	119.4 ± 7.6	64.1 ± 12.1	65.2 ± 9.5

The data were expressed by mean ± standard division (SD).

Table 7. The kinetic data of dADP substitution demonstrate the effective catalytic ability of RM-CK, RM-CK A267H, G268N, P270G and M1-CK.

	k_{cat}^{dADP} / K_m^{dADP} ($\frac{k_{cat}^{dADP}}{K_m^{dADP}}$)	
	pH 7.1	
Temperature (°C)	35.0	5.0
RM-CK	262.7 ± 33.3 1.3 ± 0.1 (195.5)	64.3 ± 6.8 1.2 ± 0.0 (54.5)
RM-CK A267H	276.9 ± 23.5 1.5 ± 0.1 (179.6)	34.8 ± 3.2 0.6 ± 0.0 (62.1)
RM-CK G268N	225.1 ± 9.8 1.1 ± 0.1 (206.6)	28.9 ± 1.5 0.4 ± 0.0 (73.3)
RM-CK P270G	301.5 ± 14.1 1.5 ± 0.1 (196.7)	19.2 ± 0.9 0.3 ± 0.0 (68.6)
M1-CK	217.8 ± 28.2 1.1 ± 0.1 (201.7)	64.3 ± 1.6 0.9 ± 0.1 (75.8)
	pH 8.0	
Temperature (°C)	35.0	5.0
RM-CK	33.5 ± 2.8 0.8 ± 0.0 (43.0)	6.3 ± 0.2 0.1 ± 0.0 (59.3)
RM-CK A267H	48.6 ± 7.4 0.3 ± 0.0 (162.2)	2.0 ± 0.2 0.1 ± 0.0 (14.8)
RM-CK G268N	39.3 ± 2.7 0.5 ± 0.1 (76.4)	2.8 ± 0.4 0.1 ± 0.0 (26.1)
RM-CK P270G	36.7 ± 2.1 0.3 ± 0.0 (123.5)	2.6 ± 0.2 0.1 ± 0.0 (24.6)
M1-CK	20.8 ± 2.2 0.3 ± 0.1 (64.6)	15.2 ± 1.8 0.2 ± 0.0 (74.9)

Table 8. The prediction m/z and observation results of RM-CK and M1-CK residue 268 mutants.

	m/z Prediction		m/z
CK mutants	nonphosporylated	monophosphorylated	observed
M1-CK	2952.36	3032.36	3031.88
M1-CK N268G	2895.31	2975.31	2975.89
M1-CK N268D	2953.35	3033.35	3033.23
M1-CK NS68K	2966.45	3046.45	3047.19
M1-CK N268L	2951.42	3031.42	3030.66
RM-CK	2871.28	2951.28	2951.41
RM-CK G268N	2928.33	3008.33	3008.63
RM-CK G268D	2929.32	3009.32	3009.78
RM-CK G268K	2743.15	2823.15	2823.16
RM-CK G268L	2927.39	3007.39	3007.54

Table 9. The distance of Cα between selected residues and residue 268 and their solvent accessible surface area of RM-CK and RM-CK G268N.

	Cα distance (Å)		SAS (Å^2)	
	RM-CK	RM-CK G268N	RM-CK	RM-CK G268N
Cys^{283}	24.07	23.86	38.2 (15.6%)	35.7 (15.0%)
Thr^{103}	28.27	28.27	106.6 (43.9%)	105.7 (43.0%)
Arg^{148}	19.94	20.16	126.4 (37.0%)	122.2 (36.1%)
Arg^{152}	16.34	16.61	122.5 (34.7%)	119.0 (34.3%)
Glu^{160}	14.13	12.91	101.1 (35.4%)	90.4 (31.0%)
Asn^{163}	18.23	16.07	97.9 (37.3%)	64.6 (24.7%)
Lys^{170}	23.25	21.79	98.0 (32.5%)	93.6 (32.4%)
Lys^{172}	23.41	23.01	100.3 (33.0%)	113.8 (38.1%)
Lys^{177}	27.77	26.33	135.4 (46.2%)	128.4 (43.1%)
Ser^{178}	29.47	28.91	66.6 (30.1%)	61.5 (27.8%)
Glu^{183}	33.08	32.03	44.4 (15.3%)	39.1 (13.8%)

APPENDIX

Appendix A. The Carp M1 Muscle-specific creatine kinase subisoform is adaptive to the synchronized changes in body temperature and intracellular pH that occur in the common carp.

Appendix B. Activity function of rabbit muscle-specific creatine kinase at low temperature by mutation at Gly268 to Asn268.

Appendix A.

Wu, C.L., Liu, C.W., Sun, H.W., Chang, H.C., Huang, C.J., Hui, C.F. and Wu, J.L.

The Carp M1 Muscle-specific creatine kinase subisoform is adaptive to the synchronized changes in body temperature and intracellular pH that occur in the common carp.

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The carp M1 muscle-specific creatine kinase subisoform is adaptive to the synchronized changes in body temperature and intracellular pH that occur in the common carp *Cyprinus carpio*

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The three previously cloned *Cyprinus carpio* muscle-specific subisoforms of creatine kinase (CK, EC 2.7.3.2) designated M1-, M2- and M3-CK were examined. At temperatures <15° C and at pH >7·7, specific activities of M1-CK were three to eight-fold higher than specific activities of M3- and rabbit (R) M-CK. At pH 8·0, M1-CK exhibited its highest specific activity at 15° C. Michaelis constants of PCr (K_m^{PCr}) and ADP (K_m^{ADP}) of M1-CK were relatively stable at pH between 7·1–8·0 and 25–5° C. Its calculated activation energy of catalysis (E_a) at pH 8·0 was lower than at pH 7·1. Circular dichroism spectroscopy results showed that changes in secondary structures in M1-CK at the pH and temperatures studied were much less than in the cases of RM- and M3-CK. The M1-CK enzyme seemed to have evolved to adapt to the synchronized changes in body temperature and intracellular pH of *C. carpio*.

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Key words: acclimation; carp; creatine kinase; kinetics; pH; secondary structure.

INTRODUCTION

Habitat temperature of the eurythermic common carp *Cyprinus carpio* (L.) varies from 5 to >35° C (Hwang & Lin, 2002; Stecyk & Farrell, 2006). It has been known that the pH of extracellular and intracellular fluids of poikilothermic animals varies inversely with body temperature (Yancey & Somero, 1978). In *C. carpio*, blood and intracellular pH rises from 7·6 to 8·0 when water temperature decreases from 20 to 10° C, and it could change 0·4 pH U within a day (Reeves, 1977; Rothe & Heisler, 1979; Albers *et al.*, 1983; Moyes *et al.*,

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1988). Thus, there is interest in many aspects of their physiological responses such as metabolism (Wodtke, 1981; Guderley, 1990) neuron response (Ven der Linden *et al.*, 2004), endocrine release (Flik *et al.*, 2006) and behaviour (Rome *et al.*,1990; Johnston & Temple, 2002; Guderley, 2004; Ven der Burg *et al.*, 2006). At this broad range of temperatures, ATP production in energy demanding cells is an important criterion to maintain *C. carpio*.

Creatine kinase (CK, EC 2.7.3.2) is one of the most important enzymes involved in energy homeostasis, and there are four different isozymes in this enzyme family. The muscle form CK (M-CK) is specific for differentiated muscle, whereas the brain form CK (B-CK) is found in brain and a variety of other tissues, and these cytosolic forms are used mostly in providing ATP for muscle contraction (Schlegel *et al.*, 1988; Qin *et al.*, 1998). Phosphagen kinases exhibit different biochemical characteristics under different temperatures Michaelis constant of ATP (K_m^{ATP}) of arginine kinase of European lobster *Homarus gammarus* has been reported to show no difference between 20 and −25° C, while using a mitochondrial respiration assay, K_m^{ADP} of tortoise CK decreased at low temperature (Travers *et al.*, 1978; Birkedal & Gesser, 2004). Recently, the M-CK of *Chaenocephalus aceratus* (Lömberg) has been found to function best at 0·5° C (Winnard *et al.*, 2003). Biochemical kinetic properties, crystallographic analysis and cryoenzymology of phosphagen kinases have been studied extensively, yet, how CK functions in order for eurythermic fishes to adapt to different ambient temperature remains to be elucidated (Travers & Barman, 1995; McLeish & Kenyon, 2005).

Previously, three *C. carpio* M-CK subisoforms, M1-, M2- and M3-CK, have been cloned (Sun *et al.*, 1998). Immunoblot and immunohistochemical analyses show that all three M-CKs were detected in red and white muscle of different temperature (30–10° C) acclimatized *C. carpio* (Sun *et al.*, 2002). It would be interesting to elucidate the possible physiological functions of the three *C. carpio* subisoforms by studying the temperature and pH-dependent changes of their biochemical properties. Because M-CK is closely related to muscle function, assay of CK has become an important clinical diagnosis. Assay of CK for disease diagnoses include cardiac diseases (Wu, 2005), muscular dystrophy (Hsu, 2004) and inflammatory responses (Nirmalananthan *et al.*, 2004). Biochemical analysis of serum CK activity has been developed since 1965, and most of these methods involve the assay of coupled enzymes or measurement of proton formation (Holliday *et al.*, 1965). Under variable temperature and pH conditions, these methods would not be suitable to determinate the specific activities of the subisoforms of *C. carpio* M-CK (Tanzer & Gilvarg, 1959; Mahowald *et al.*, 1962; Rosalki, 1967). In this study, using a modified M-CK activity assay method originally designed by Hughes, (1962), the specific activities of the rabbit *Oryctolagus cuniculus* M-CK (RM-CK), and *C. carpio* M1-, M2- and M3-CK at temperatures from 0 to 35° C, and at pH that resemble the changes in *C. carpio* intracellular pH were compared (7·1–8·0) (Hughes, 1962). Also, thermal stability of the RM- and *C. carpio* M1-, M3-CK were compared by preincubating these enzymes at temperatures from 10 to 70° C, at pH 7·1 or 8·0 for 30 min then assayed at 30° C for 15 min.

Circular dichroism (CD) spectroscopy has been employed to study the impact of intra-subunit domain–domain interactions on RM-CK, thus, here

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CD was employed to monitor changes in secondary structures of rabbit and *C. carpio* M-CKs at different pHs and temperatures (Zhao *et al.*, 2006*a*, *b*). Comparison of the results of specific activities, kinetic variables and changes in secondary structures of these three M-CKs suggests that M1-CK could play a major role in satisfying physiological demands in *C. carpio* muscle from 35 to 5° C.

MATERIALS AND METHODS

SAMPLE PREPARATION

Cyprinus carpio M1-, M2- and M3-CK cDNAs were constructed as described previously (Sun *et al.*, 1998). M1-, M2- and M3-CK recombinant plasmids (pET28a) were transformed to *Escherichia coli* BL21(DE3)pLysS and cultured in 1 l LB medium (pH 7·6). After induction, the culture medium was harvested and the pellet resuspended in binding buffer (5 mM imidazole, 0·5 M NaCl, 20 mM Tris–HCl and pH 7·9). After sonication and centrifugation at 12 000 *g*, 15 min, the supernatant was filtered using a 0·45 μm filter and applied into a nickel column. The recombinant protein was eluted with elution buffer (0·2 M of imidazole, 0·5 M of NaCl, 20 mM of Tris–HCl and pH 7·9). Eluate was dialysed in 30 mM of Tris–HCl, 10 mM of $MgCl_2$, 1 mM of β-mercaptoethanol, pH 7·1 at 4° C for 1 day and concentrated with an Amicon Ultra 15 filtration tube (10 000 mwco; Millipore Corp., Billerica, MA, U.S.A.). The concentrated protein solution was stored at −30° C with glycerol (protein solution:glycerol, v/v, 4:1). Protein concentration was estimated using the Commassie plus™ protein assay reagent (Bio-Rad, Hercules, CA, U.S.A). RM-CK was a commercial product (Merck, Darmstadt, Germany) and this enzyme was dissolved in 30 mM of Tris–HCl, 10 mM of $MgCl_2$, 1 mM of β-mercaptoethanol, pH 7·1 and stored at −30° C with glycerol (protein solution:glycerol, v/v, 4:1).

ACTIVITY ASSAY

M-CK activity assay was based on the method by Hughes (1962) and modified to fit different assay conditions. Because the Sigma M-CK activity assay kit (SIGMA Diagnostic, No. 520; Sigma-Aldrich, St Louis, MO, U.S.A.) that was used in previous studies (Sun *et al.*, 1998), was no longer available, $MgCl_2$, HCl, ADP (potassium salt), and phosphocreatine (PCr, disodium salt) were purchased from Merck; *p*-hydroxylmercuribenzoate, α-naphthol and diacetyl were from Sigma-Aldrich; Tris was from Boehringer, Ingelheim, Germany; 1,4-dithiothreitol (DTT) was from Amresco, Solon, OH, U.S.A. All chemicals were of analytical or biotech grade, and all the stock solutions were prepared as mentioned in the Sigma assay kit. The basal reaction buffer of M-CK activity assay was 30 mM of Tris–HCl, 10 mM of $MgCl_2$ and 1 mM of DTT. The pH of the assay buffers were adjusted with 1 M of HCl at the assay temperatures. M-CK activity assays were carried out at pH 7·1, 7·4, 7·7 or 8·0 and at temperatures 35, 30, 25, 20, 15, 10, 5 or 0° C. Assay solution also contained 12·5 mM of PCr, 1 mM of ADP and enzyme concentrations in the range of 0·0125–0·05 ng in 80 μl reaction volume. After 15 min of enzyme reaction, *p*-hydroxyl mercuribenzoate (50 mM, 20 μl) was added to terminate the reaction, then α-naphthol (2%, w/v; 100 μl) and diacetyl (1:200, v/v; 100 μl) were added for the colorimetric reaction, and spectrophotometric absorption was measured at 520 nm. Creatine (Cr) produced in each reaction was quantified using a Cr standard curve. Enzyme specific activity U is defined as 1 μmol of Cr formed min^{-1} mg^{-1} of enzyme.

Thermal stability experiments were carried out in the activity assay buffer at pH 7·1 or 8·0 with enzyme concentration at 0·0025 μg μl^{-1}. The assay reaction was preincubated in the absence of ADP and PCr at 10, 20, 30, 40, 50, 60 or 70° C for 30 min, then, cooled on ice before ADP and PCr were added and assayed at 30° C for 15 min.

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At least three repeats were carried out, and the highest specific activity was taken as 100% (Zhao *et al.*, 2006*a*). The specific activities of all the M-CKs in each condition were assayed from three different batches of recombinant protein preparation and each preparation assayed for more than three times.

KINETIC ANALYSIS

Kinetic analyses of M-CKs were carried out as described by Cleland (1979). For biochemical kinetic analyses, the same specific activity assay mentioned above was carried out at pH 7·1 or 8·0, and at 25, 15 or 5° C. ADP concentrations ranged from 0·1 to 1 mM with PCr concentration at 12·5 mM for RM-CK, and 100 mM for M1- and M3-CKs when determining Michaelis constant of ADP (K_m^{ADP}). PCr concentrations ranged from 0·5 to 100 mM with ADP concentration at 1 mM when determining Michaelis constant of PCr (K_m^{PCr}), and Specificity constant (k_{cat}), K_m^{ADP} and K_m^{PCr} were calculated using the Michaelis–Menten equation (Marangoni, 2003), $v = V_{max}(S)[K_m + (S)]^{-1}$, where v was reaction rate obtained from Cr formation rate, (S) was the initial concentration of one of the substrates, while the other substrate concentration was fixed, V_{max} is defined as the maximum reaction velocity, $V_{max} = k_{cat}(E_{total})$, where E_{total} = total enzyme concentration. In the steady-state case, $v^{-1} = K_m(S)^{-1}k_{cat}^{-1}(E_{total})^{-1} + k_{cat}^{-1}(E_{total})^{-1}$, and the graph v^{-1} *v.* $(S)^{-1}$ was plotted. On the graph, when $(S)^{-1} = 0$, $v^{-1} = k_{cat}(E_{total})^{-1}$, and when $v^{-1} = 0$, $(S)^{-1} = -K_m^{-1}$.

K_m and k_{cat} were obtained using these formulae. Reaction time was 7 min for the 25° C, 15 min for the 15° C and 45 min for the 5° C reactions. Reaction rates were determined as above. All the results were repeated with different batches of recombinant protein. The molecular masses were calculated using the free website ExPASy Proteomics Server (http://tw.expasy.org/tools/pi_tool.html), and concentrations of the enzymes were calculated using these data.

CIRCULAR DICHROISM SPECTROSCOPY

Circular dichroism spectra were recorded on a Jasco J715 spectropolarimeter (Jasco International, Tokyo, Japan) at temperatures 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50° C (Kelly *et al.*, 2005). For far-UV spectrum, 1 mm path-length quartz cuvette of 200 μL was used at a scan speed of 20 nm min^{-1} from 200 to 250 nm. The CD solution contained 2·32 μM of M-CK protein in the activity assay buffer at pH 7·1 or 8·0 in the absence of ADP and PCr. Data were collected per 0·1 nm and an average of 10 spectra was corrected by subtraction of spectra recorded on the activity assay buffer in the absence of enzyme. Raw data were smoothed by moving average of 19 consecutive data points.

RESULTS

M1-CK WAS MORE ACTIVE ≤30° C THAN RM-, M2- AND M3-CK

The assay condition at pH 7·1 that allowed M1-CK to exhibit highest specific activity at 30° C was taken as the basal condition. Sodium chloride and potassium chloride reduced M1-CK activity even at 5 mM. RM-, M1-, M2- and M3-CK activities were assayed at four different pH and eight different temperatures. RM-CK showed its highest specific activity (mean ± S.D.) of 327·5 ± 8·2 U at pH 7·1 and 35° C [Fig. 1(a)]. Its activity started to decrease at temperatures <35 and >35° C. M1- and M3-CK showed their highest activities (mean ± S.D.) of 287·1±15·0 and 142·6 ±16·6 U, respectively, at pH 7·1, 30° C. M2-CK

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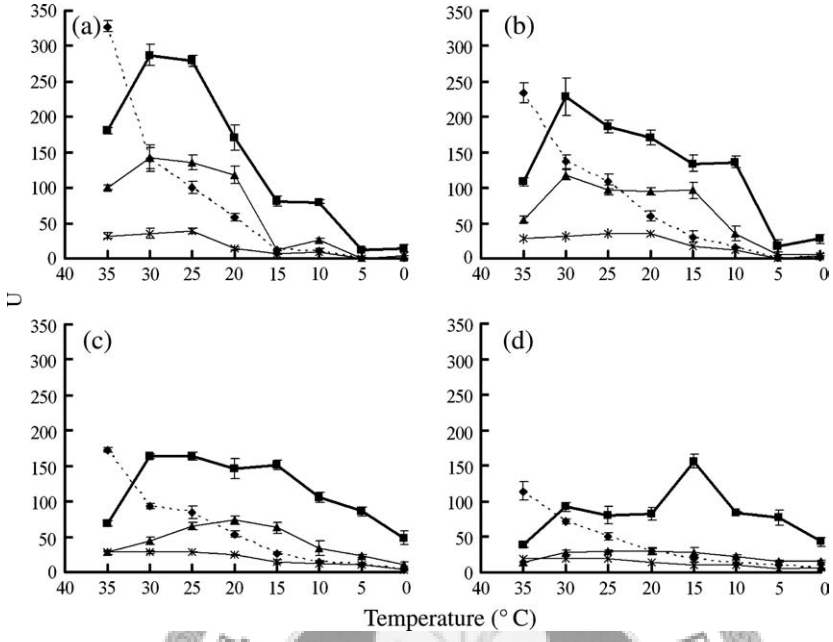

FIG. 1. Mean ± S.D. specific activities of rabbit (RM-; ◆) and *Cyprinus carpio* [M1- (■), M2- (✻) and M3- (▲)] muscle-specific creatine kinase (CK) at different temperatures and pH. At least three replicates from three batches of recombinant protein preparation were conducted in each condition. The product creatine (Cr) was reacted with α-naphthol and diacetyl, and the colour product absorbance was measured at 520 nm. The blank reaction without enzyme was subtracted from the absorption values. Buffer pH (a) 7·1, (b) 7·4, (c) 7·7 and (d) 8·0. U, 1 μmol of Cr formed min^{-1} mg^{-1} of enzyme.

specific activities, however, were <40 U at all pH and temperatures tested, therefore, its biochemical characteristics were not pursued further. At pH 7·1, 15° C, RM- and M3-CK lost >90% of their enzymatic activities, while M1-CK lost 73%. M1-CK, however, seemed to be able to maintain its activity between 15 and 10° C before it suffered a further loss at 5° C.

At pH 7·4, 30° C, activities of all three enzymes decreased slightly [Fig. 1(b)]. Activity of M3-CK did not change between 25 and 15° C, while activity of M1-CK also showed a plateau between 20 and 10° C. At pH 7·7, M1-CK activity remained stable from 30 to 15° C, while activity of M3-CK increased from 30 to 20° C then decreased when below 20° C [Fig. 1(c)]. At pH 8·0, activities of RM- and M3-CK were significantly lower, yet M1-CK activity remained stable from 30 to 20° C, then increased significantly at 15° C [Fig. 1(d)]. M1-CK was able to maintain its activity around 150 U at 15° C, and even at 0° C, its activity of almost 50 U was 60% the M-CK activity of *C. aceratus* at 0° C (Winnard *et al.*, 2003). Therefore, it seems that M1-CK can function well in *C. carpio* muscle from 35 to 5° C.

CHANGES IN K_m^{PCr} AND K_m^{ADP} OF M1-CK AT DIFFERENT TEMPERATURES

Since the differences in activities between RM-, M1- and M3-CK were most significant at pH 7·1 and 8·0, and M1-CK showed interesting activity

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feature at pH 8·0, 15° C, determination of K_m^{PCr} and K_m^{ADP} was carried out at these two pHs and at temperatures 25, 15 and 5° C. At pH 7·1, 25° C, K_m^{PCr} of RM-, M1- and M3-CK were 1·25 ± 0·02, 6·32 ± 0·72 and 7·70 ± 0·51 mM (mean ± S.D.), respectively [Fig. 2(a)]. K_m^{PCr} of RM- and M1-CK increased when temperature decreased from 25 to 5° C. In the case of M3-CK, K_m^{PCr} from 25 to 15° C was at similar range to the other two enzymes then climbed steeply from 15·17 ± 0·99 mM at 15° C to 76·91 ± 3·88 mM (means ± S.D.) at 5° C. This implied a significant conformational change in the protein structure of the M3-CK. At pH 8·0, when temperature decreased from 25 to 5° C, K_m^{PCr} of RM-CK and M3-CK decreased slightly, while the M1-CK values seemed unchanged [Fig. 2(b)]. At pH 7·1, K_m^{ADP} of RM-CK decreased steeply from 25 to 15° C then levelled off from 15 to 5° C, and M3-CK increased from 25 to 15° C then levelled off from 15 to 5° C [Fig. 2(c)]. At this pH, the values of K_m^{ADP} of M1-CK changed little throughout. At pH 8·0, from 25 to 5° C, the changes in K_m^{ADP} of M3-CK were similar to those at pH 7·1, while K_m^{ADP} of RM- and M1-CK varied little throughout [Fig. 2(d)].

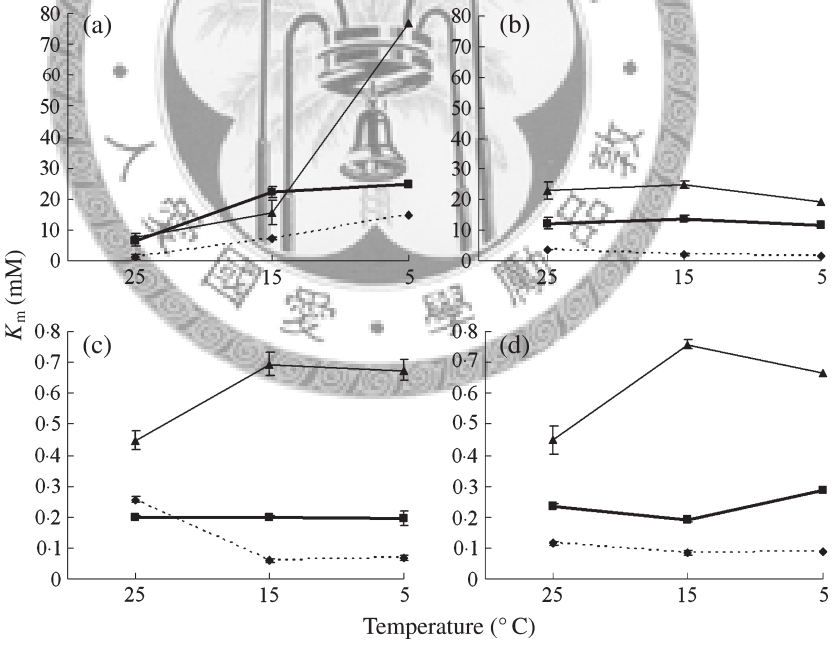

FIG. 2. *Cyprinus carpio* mean ± S.D. Michaelis constant (K_m) of RM- (◆), M1- (■) and M3- (▲) CK (see Table I) at different temperatures and pHs. K_m values were derived from double-reciprocal plots of the Michaelis–Menten equation. At least three replicates from three batches of recombinant protein preparation were conducted in each condition. The blank reaction without enzyme was subtracted from the absorption values. Michaelis constant of PCr (K_m^{PCr}) at (a) pH 7·1, (b) pH 8·0; ADP (K_m^{ADP}) at (c) pH 7·1, (d) pH 8·0 (PCr, phosphocreatine).

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SPECIFICITY CONSTANT ($K_{CAT}\ K_M^{-1}$) AND ACTIVATION ENERGY OF CATALYSIS (E_A) OF RM-, M1- AND M3-CK

Due to high K_m^{PCr} of the three enzymes at low temperature, it was impossible to carry out specific activity assay for k_{cat} calculation at $5 \times K_m^{PCr}$. Therefore, the highest concentration of PCr was set at 100 mM, which was higher than intracellular concentration of PCr (Iyengar *et al.*, 1985). k_{cat} calculation showed that at both pH 7·1 and 8·0, from 25 to 5° C, the k_{cat} values of RM-CK were more affected by decrease in temperature, while at pH 8·0, the k_{cat} values of M1-CK decreased least (Table I).

Catalytic efficiency $[k_{cat}(K_m^{PCr})^{-1}]$ of the three enzymes decreased when temperature decreased (Table I). The $k_{cat}(K_m^{PCr})^{-1}$ of M1-CK did not show any advantage over RM- and M3-CK, except at 5° C. At pH 7·1, between 25 and 15° C, $k_{cat}(K_m^{ADP})^{-1}$ showed that M3-CK seemed to be the most efficient enzyme among the three enzymes. Curiously, the highest $k_{cat}(K_m^{ADP})^{-1}$ of RM-CK was at 15° C. Though at both pHs, $k_{cat}:(K_m^{ADP})^{-1}$ of all three enzymes decreased as temperature decreased, the decrease in catalytic efficiency of M1-CK seemed to be the most gentle.

TABLE I. Biochemical kinetic values of muscle-specific subisoforms of creatine kinase (CK) of rabbit (RM-CK) and *Cyprinus carpio* (M1- and M3-CK) at different temperatures and pH. Specificity constants (k_{cat}) values (means ± S.D.) were derived using the Michaelis–Menten equation. k_{cat} per Michaelis constant of PCr (K_m^{PCr}) and k_{cat} per Michaelis constant of ADP (K_m^{ADP}) were calculated using the experimental data in Fig. 2

Assay	Temperature (° C)	k_{cat} ($\times 10^3$ s^{-1})	$k_{cat}(K_m^{PCr})^{-1}$ ($\times 10^3$ mM^{-1} s^{-1})	$k_{cat}(K_m^{ADP})^{-1}$ ($\times 10^3$ mM^{-1} s^{-1})	E_a (kJ mol^{-1})
pH 7·1					
	25	8·31 ± 0·47	6·66	30·61	
RM-CK	15	4·01 ± 0·49	0·62	69·04	63·80
	5	1·31 ± 0·16	0·09	19·07	
	25	17·82 ± 2·09	2·82	46·83	
M1-CK	15	11·85 ± 2·12	0·56	34·18	39·70
	5	5·65 ± 0·72	0·26	15·23	
M3-CK	25	35·60 ± 1·17	4·62	77·61	79·82
	15	13·76 ± 1·37	0·91	64·09	
	5	3·52 ± 0·72	0·05	17·62	
pH 8·0					
	25	4·66 ± 0·93	1·25	42·66	
RM-CK	15	1·80 ± 0·16	0·93	21·04	64·41
	5	0·72 ± 0·12	0·42	8·10	
	25	7·78 ± 0·56	0·64	35·64	
M1-CK	15	6·00 ± 0·47	0·43	34·90	27·05
	5	3·56 ± 0·22	0·30	20·95	
	25	12·42 ± 0·56	0·54	35·84	
M3-CK	15	3·48 ± 0·76	0·16	25·23	58·23
	5	2·27 ± 0·27	0·13	13·85	

E_a, Activation energy of catalysis.

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Biochemical kinetic properties of M1-CK were quite different from those of RM- and M3-CK (Fig. 2 and Table I). K_m^{PCr}, K_m^{ADP} and k_{cat} of M1-CK varied least at different pHs and temperatures. K_m^{PCr} and K_m^{ADP} of M3-CK were all higher than those of the RM-CK under all pH and temperature conditions in the present study. Especially at pH 7·1, at 5° C, K_m^{PCr} of M3-CK was >3·5-folds higher than K_m^{PCr} of RM- and M1-CK. Since K_m^{ADP} of all three enzymes were relatively stable, and all $k_{cat}(K_m^{ADP})^{-1}$ were much higher than all $k_{cat}(K_m^{PCr})^{-1}$ in all three enzymes, ADP binding did not seem to be the rate limiting factor in M-CK catalysis at different pHs and temperatures (Table I). When comparing the highest $k_{cat}(K_m^{PCr})^{-1}$ at pH 7·1, 25° C to the lowest at pH 8·0, 5° C, of all three enzymes, the decrease in $k_{cat}(K_m^{PCr})^{-1}$ were 9·4-folds in M1-CK, 15·9-folds in RM-CK and 27·2-folds in M3-CK.

Activation energy of catalysis (E_a) was calculated using the Arrhenius equation (Lonhienne *et al.*, 2000), $k_{cat} = Ae^{-E_a(RT)^{-1}}$, where $-E_a R^{-1}$ is the slope of the Arrhenius plot when $\ln k_{cat}$ is plotted as a function of temperature (T^{-1}). R is the gas constant (8·314 J mol^{-1} K^{-1}). E_a values (kJ mol^{-1}) of M1-CK were the lowest among the three enzymes at both pH values (Table I). E_a values of RM-CK were *c.* 64 kJ mol^{-1} at both pH values, which reflected that E_a of RM-CK was not affected by pH. The E_a values of both M1- and M3-CK, however, decreased when pH changed from 7·1 to 8·0. Therefore, M1- and M3-CK of *C. carpio* were more affected by pH.

THERMAL STABILITY OF THE THREE M-CKS AND MONITORING OF THEIR SECONDARY STRUCTURE CONTENT WITH CD SPECTROSCOPY

A CD signal will be observed when a chromophore is placed in an asymmetric environment by virtue of the three-dimensional structure adopted by the molecule, and CD spectrum is obtained when the dichroism is measured as a function of wavelength. In protein, different types of secondary structural elements such as α-helices, β-sheets, β-turns and random coil induce bands of distinctive shapes and magnitudes in the far-UV. For example, α-helix induces an intense positive band at 190 nm and a negative band at 208 and 220 nm. Therefore, the loss of CD signals either on addition of denaturing agents or by an increase in temperature can be used to provide quantitative estimates of the stability of the folded state of the native protein (Kelly *et al.*, 2005).

Comparisons of thermal stability of the three M-CKs showed that RM-CK was indeed the most thermal stable and M3-CK the least (Fig. 3). Both RM- and M3-CK were rendered less thermal stable at pH 8·0, though by different extent. For M3-CK, even preincubation at 20 and 30° C caused a significant decrease in its activity. At pH 7·1, after preincubation at 40° C, M1-CK retained over 60% its highest activity, and at pH 8·0, 50%. Also, thermal stability of M1-CK did not differ significantly at either pH.

The far-UV CD spectra (200–250 nm) of RM-, M1- and M3-CK are shown in Fig. 4. It revealed that the secondary structures (α-helix and β-sheet) of RM- and M3-CK fluctuated significantly at both pH values and at different temperatures. While, the overall CD profiles of M1-CK varied little from 40 to 5° C,

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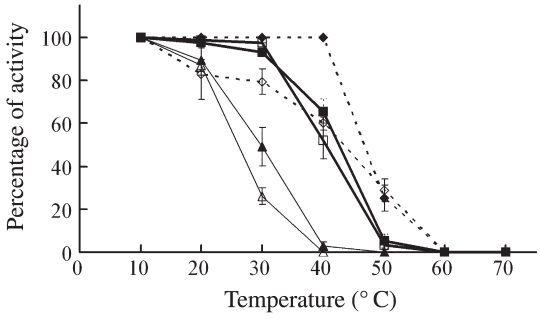

FIG. 3. Thermal inactivation assays at pH 7.1 of RM- (◆), M1 (■) and M3-CK (▲); and pH 8·0 of RM-(◇), M1-(□) and M1(△) for *Cyprinus carpio* (see Table I) in relation to temperature. The assay reaction was preincubated in the absence of ADP and PCr at various temperatures for 30 min, cooled on ice before ADP and PCr were added and assayed at 30° C for 15 min. Aleast three replicates of three batches of recombinant enzymes were conducted for each assay. Values are means ± S.D.

it only started to denature at 45° C. Taken together, these results show that M1-CK is more adaptive to the synchronized changes in body temperature and intracellular pH that occur in *C. carpio*.

DISCUSSION

Environmental temperature directly influences the extracellular and intracellular pH of ectothermic fishes, subsequently intracellular pH influences the functions of all enzymes (White & Somero, 1982). In this study, the possible physiological function of M1-, M2- and M3-CK was studied by comparing their biochemical kinetic features with RM-CK at pH that resemble the variation in intracellular pHs in *C. carpio* at different temperatures (Fig. 1). At pH 8·0, 15° C, only M1-CK was able to maintain its specific activity at >50% (156 U) its highest specific activity (287 U, at pH 7·1, 30° C), while specific activity of M3-CK decreased to 25 U. These results suggest that, in *C. carpio* muscle, M1-CK alone can play a major role in maintaining energy homeostasis at various water temperatures.

In earlier studies, no change was observed in the percentages of mRNA and protein content of the three subisoforms of *C. carpio* M-CKs under different water temperatures, thus, the adaptive strategy of the M-CK enzymes towards varible environmental temperatures would probably be at the enzyme activity level (Sun *et al.*, 2002; Andrew *et al.*, 2004). In a previous study, M3-CK homodimer could not be detected at temperature >20° C, pH 7·4, yet in the present study, in the modified assay buffer, at pH from 7·1 to 7·7, and at temperatures <30° C, specific activities of M3-CK are comparable to those of RM-CK (Sun *et al.*, 2002). This discrepancy could be due to that the previous dimerization experiment of M3-CK was being carried out in PBS which was devoid of Mg^{2+}, while the present assay buffer contained 10 mM of $MgCl_2$ and 1 mM of DTT and both might help in the stabilization of M3-CK dimer at temperatures >20° C. Specific activities of M1- and M3-CK are much higher in the present modified buffer than when the commercial M-CK assay kit was used in the previous study (Sun *et al.*, 1998).

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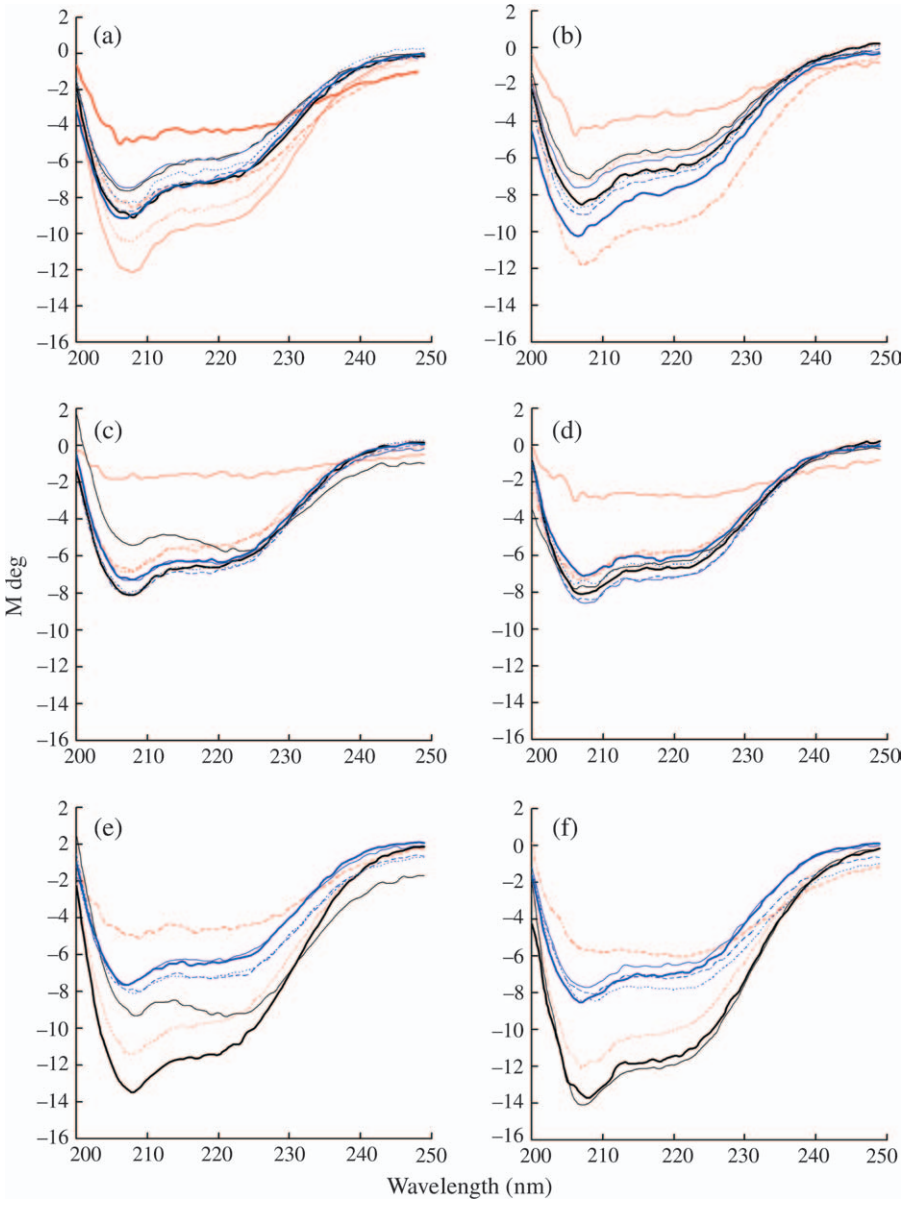

FIG. 4. Spectra of far-UV circular dichroism (CD) spectroscopy of (a) RM-CK pH 7·1, (b) RM-CK pH 8·0, (c) M1-CK pH 7·1, (d) M1-CK pH 8·0, (e) M3-CK pH 7·1 and (f) M3-CK pH 8·0 (see Table I) of *Cyprinus carpio* at different temperatures (50; 45; 40; 35; 30; 25; 20; 15; 10; and 5° C].

At low temperature, k_{cat} in a psychrophilic enzyme is generally higher than in a mesophilic enzyme, and E_a of the psychrophilic enzyme decreases to favour k_{cat} (Lonhienne *et al.*, 2000). E_a values of M1-CK were the lowest among the three enzymes both at pH 7·1 and 8·0, furthermore, the E_a value of M1-CK

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at pH 8·0 was lower than that at pH 7·1 (Fig. 3 and Table I). This unique functional property of M1-CK, relatively stable K_m^{PCr} and $K_m^{ADP}$and lower E_a, suggests that M1-CK has evolved to become an enzyme that can function between pH 7 and 8 and at temperatures from 35 to 5° C (Cook *et al.*, 1981).

An advantage of CD spectroscopy is that it enables the monitoring of dynamic changes in solution in the secondary structures of RM-, M1- and M3-CK at different pH and temperatures (Kelly *et al.*, 2005). Changes in secondary structures of M1-CK at the pH and temperatures under studied were much less than in the cases of RM- and M3-CK. For M3-CK, at 30° C, its CD spectra at both pH show that its secondary structures started to deviate from the 25° C profile. Crystal structures of human and RM-CKs have been determined (Rao *et al.*, 1998; Shen *et al.*, 2001; Ohren *et al.*, 2007). The high-resolution RM-CK structure shows that Asp^{55} in the RM-CK-ADP subunit forms a hydrogen bond to the side chain of His^7 in the adjacent RM-CK-TSA subunit, while Asp^{55} from the RM-CK-TSA subunit forms a hydrogen bond to the backbone N atom of Pro^2 within the same chain (Ohren *et al.*, 2007). It has been proposed that the loss of this hydrogen bond was sufficient to cause dissociation of the dimer. In the case of M3-CK, both the Pro^2 and His^7 are absent in the N-terminal, thus, it might be the reason for the thermal instability of this enzyme (Sun *et al.*, 1998).

It has been suggested that a 16° C range of temperatures cannot be covered by one metabolic profile (Guderley, 1990). Indeed, myofibrillar heavy chain (MHC) isoform expression can be modified by thermal acclimation, and production of biochemically different myofibrillar ATPase in white muscle can response to environmental temperature (Gerlach *et al.*, 1990; Johnston *et al.*, 1990). More recently, cold induced gene expression has been found to include acyl-CoA Δ9-desaturase, RNA polymerase II activators, RNA helicase, TCP1 chaperonins, and a large group of genes involved in ubiquitin-dependent protein catabolism and proteasomal function (Gracey *et al.*, 2004). In the case of *C. carpio* M-CKs, a study using 2-D gel on cold acclimation and expression of soluble proteins in *C. carpio* skeletal muscle revealed a downregulation of expression of M2- and M3-CK, and an increased accumulation of their fragments (McLean *et al.*, 2007). Taken together, M3-CK is thermal unstable as well as subject to ubiquitination and proteolytic fragmentation at low temperature. And, since M2-CK is much less active, the proposal that M1-CK is the only one that functions well at pH 7–8, and from 35 to 5° C is strengthened. Of course, post-translational regulation of the carp M-CKs at different temperatures cannot be ruled out, but at least it was not observed in the 2-D gel studies (McLean *et al.*, 2007)

In this study, the biochemical characteristics, thermal stability and CD spectroscopy analyses of two muscle-specific subisoforms of CK of the eurythermic *C. carpio*, and RM-CK of an endothermic animal were compared. In conclusion, the M1-CK is the only one that functions well at pH from 7 to 8 and from 35 to 0° C. And, since selection pressure on each *C. carpio* muscle-specific subisoform would be less than if there were only one muscle-specific isoform, M1-CK subisoform seems to be the only one that has evolved to become adaptive to the synchronized changes in body temperature and intracellular pH that occur in *C. carpio*.

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Appendix B.

國立臺灣大學 國家動物園

Wu, C.L., Li, Y.H., Lin, H.C., Yeh, Y.H., Yan, H.Y., Hsiao, C.D., Hui, C.F. and Wu, J.L.

Activity function of rabbit muscle-specific creatine kinase at low temperature by mutation at Gly268 to Asn268.

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Activity and function of rabbit muscle-specific creatine kinase at low temperature by mutation at gly^{268} to asn^{268}

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ABSTRACT

Carp muscle-specific creatine kinase M1 isoenzyme (M1-CK) seems to have evolved to adapt to synchronized changes in body temperature and intracellular pH. When gly^{268} in rabbit muscle-specific creatine kinase was substituted with asn^{268} as found in carp M1-CK, the rabbit muscle-specific CK G286N mutant specific activity at pH 8.0 and 10 °C was more than 2-fold higher than that in the wild-type rabbit enzyme. Kinetic studies showed that K_m values of the rabbit CK G268N mutant were similar to those of the wild-type rabbit enzyme, yet circular dichroism spectra showed that the overall secondary structures of the mutant enzyme, at pH 8.0 and 5 °C, were almost identical to the carp M1-CK enzyme. The X-ray diffraction pattern of the mutant enzyme crystal revealed that amino acid residues involved in substrate binding are closer to one another than in the rabbit enzyme, and the cysteine283 active site of the mutant enzyme points away from the ADP binding site. At pH 7.4–8.0 and 35–10 °C, with a smaller substrate, dADP, specific activities of the mutant enzyme were consistently higher than the wild-type rabbit enzyme and more similar to the carp M1-CK enzyme. Thus, the smaller active site of the RM-CK G268N mutant may be one of the reasons for its improved activity at low temperature.

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1. Introduction

Creatine kinase (CK, EC 2.7.3.2) plays an important role in the homeostasis of high energy phosphate compounds. Muscle-specific creatine kinase (M-CK) catalyses the reversible reaction that converts phosphocreatine (PCr) and ADP to creatine (Cr) and ATP and thus provides ATP for muscle contraction (Wyss et al., 1991; McLeish and Kenyon, 2005). CKs are highly conserved in vertebrates. The M-CK of endothermic rabbit *Oryctolagus cuniculus* (RM-CK) has been cloned and its crystal structure resolved (Putney et al., 1984; Rao et al., 1998). The specific activity of RM-CK is highest at around 35 °C to 40 °C, and then decreases as temperature decreases.

Ectothermic animals such as the common carp (*Cyprinus carpio*), are required to maintain physiologic and metabolic capabilities when the temperature of their habitat varies over large temperature ranges. The temperature of the habitat of the carp can vary from 5 °C to 35 °C (Stecyk and Farrell, 2006).

Previously, three *C. carpio* CK isoforms (M1-, M2- and M3-CK) were cloned (Sun et al., 1998). The overall activity of M2-CK was found to be very low, and both M2-CK and M3-CK were both found to be thermally unstable as well as subject to ubiquitination and proteolytic fragmentation at low temperature (McLean et al., 2007; Wu et al., 2008). Meanwhile, within the range of intracellular pH variation (pH 7–8) that occurs in *C. carpio* when ambient and body temperature changes, CK shows comparable specific activity to rabbit RM-CK at pH 7.1, 30 °C, as well as able to maintain 50% of its highest specific activity at pH 8, 15 °C (Wu et al., 2008). M1-CK has been suggested to have evolved to become the only CK that is able to function over the range of intracellular pH and body temperature variation that occurs in *C. carpio* (Wu et al., 2008).

The primary structures of RM-CK and carp M1-CK share 86% identity. Well conserved residues have been studied by site directed mutagenesis

Abbreviations: CK, creatine kinase; PCr, phosphocreatine; Cr, creatine; M-CK, muscle-specific creatine kinase; RM-CK, rabbit muscle-specific creatine kinase; M1-CK (M2-CK M3-CK), carp muscle-specific creatine kinase subisoform 1 (2, 3); DTT, 1,4-dithio-DL-threitol; dADP, 2′-deoxyadenosine-5′-diphosphate.

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Table 1
Crystallographic analysis.

Crystallographic data-collection and refinement statistics	
Space group	
Unit-cell parameters (Å)	I4
a = b	179.53
c	68.35
Date collection	
Wavelength (Å)	1.54
No. of reflections	62110
No. of unique reflections	16186
Average multiplicity	3.8
Completeness (%)	97.4 (92.8)*
⟨I/σ(I)⟩	21 (2.8)*
Rmerge (%)	7.6 (51)*
Resolution (Å)	50–3.3
Refinement	
Refinement program	REFMACS
Resolution range (Å)	50–3.3
Rwork/Rfree (%)	19.0/30.0

*Values in parentheses are for data in highest resolution shell.

and chemical modification (Chen et al., 1996). Arginine and histidine are thought to be important in stabilizing ADP or ATP as well as involved in acid/base catalyst generation (Cantwell et al., 2001). Tertiary and domain structures of CK have been studied on crystals of phosphagen kinases, mitochondria CK and muscle form CKs (Zhou et al., 1998; Fritz-Wolf et al., 1996; Rao et al., 1998). The N-terminal domain of CK has been shown to be the dimerization domain and the C-terminal domain is the catalytic domain that contains symmetric β-sheets (Fritz-Wolf et al., 1996; Eder et al., 1999; Rao et al., 1998). Arg^{96}, arg^{129}, arg^{131}, arg^{235}, arg^{291}, arg^{319}, and arg^{340} have been reported to be transition-state analog complex (TSAC) binding residues, and his^{61}, his^{92}, his^{186}, and his^{295} participate in catalysis (Uda et al., 2009; Jourden et al., 2005; Edmiston et al., 2001; Wood et al., 1998; Forstner et al., 1997; Chen et al., 1996; Fritz-Wolf et al., 1996). Negative residues, glu^{226}, glu^{227}, and asp^{228}, located within the active site of mitochondria CK are important for catalytic activity (Eder et al., 2000). Two flexible loops, residues 60–65 and 316–326, control the entrance of creatine or phosphocreatine, and creatine interacts with val^{71}, asp^{231} and glu^{326}, and cys^{283} is important but not essential for catalysis (Furter et al., 1993; Forstner et al., 1996; Tanaka and Suzuki 2004; Cantwell et al., 2001; Ohren et al., 2007; Reddy et al., 2003).

In M-CK, cys^{283} forms part of a conserved cysteine–proline–serine motif and its pK_a has been found to be 5.6 ± 0.1, about 3 pH units below that of a regular cysteine residue. Employing computational and UV difference spectroscopy studies of wild-type human M-CK and the P284A, S285A and C283S/S285C mutants, suggested that ser^{285} lowers the pK_a about 1 pH unit and pro^{284} lowers it a further 1 pH unit (Wang et al., 2006; Cook et al., 1981). Taken together, the change in ambient temperature affects carp body temperature and intracellular pH which in turn affects the pK_a of carp M1-CK (Albers et al., 1983). In this study we investigated the amino acid residues in M1-CK that contribute most to its cold-adaptation properties. In order not to affect the enzyme activity, residues that are different between RM-CK and M1-CK but located in secondary structures were avoided. Thus, using RM-CK cDNA as template, most mutations were made in the random coil regions and the hinge region between the N- and C-terminals. Finally, three RM-CK mutants, designated as RM-CK A267H, RM-CK G268N and RM-CK P270G, that still retained significant enzyme activity were generated and their enzymatic and physiochemical properties examined. Crystals of the mutant RM-CK G268N were successfully raised to study the fine structural change around the active site. The results of this study show that one residue mutation in RM-CK can cause the mutant RM-CK to exhibit cold-adapted enzyme properties comparable to those of *C. carpio* M1-CK.

2. Materials and methods

2.1. Construction and production of RM-CK mutants

A rabbit muscle cDNA library was purchased from Merck, and RM-CK was cloned with primer pairs 5′-atcccatatgccgttcggcaac-3′ and 5′-aaaactcgagctacttctgggc-3′. The PCR product was digested with *NdeI* and *XhoI*, and then ligated into pET 28a. Bridge PCR method was used in site directed mutagenesis on RM-CK-pET 28a clone with internal

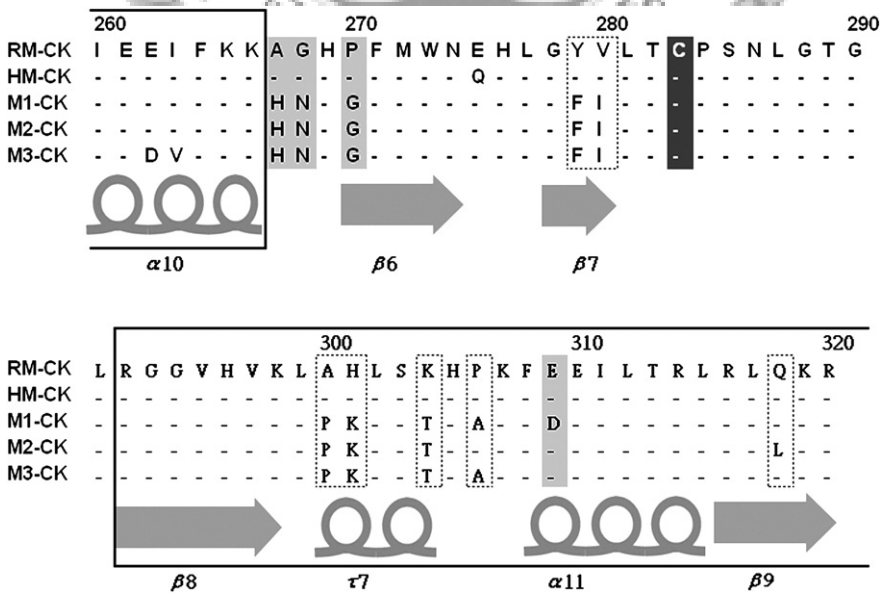

Fig. 1. Peptide sequence alignment of rabbit RM-CK, human HM-CK and carp M1, M2 and M3-CK close to the active site cysteine283. Black box indicates the key residue cys^{283} of the active site. Dotted and gray boxes are the residues that are different between RM-CK and carp M-CKs and gray boxes are the mutation sites made in this study. The α-helix (curve) and β-sheet (arrow) regions are indicated below the sequences. Black frame regions are part of the symmetric structures of the substrates binding site. The mutated residues A267H, G268N, P270G and E309D is located outside the symmetric region and any secondary structures.

primer pairs for mutation sites, 5′-tgaaggggtgcccatgtttctt-3′ and 5′-agatctttaagaaacatgggca-3′ for RM-CK A267H; 5′-cacatgaaggggtggt-tagctttc-3′ and 5′-ctttaagaaagctaaccacccc-3′ for RM-CK G268N; 5′-tcattccacatgaagccgtgcc-3′ and 5′-aaagctgggcacggcttcat-3′ for RM-CK P270G; 5′-cgggtgagaatctcgtcgaacttgggg-3′ and 5′-ccccaagttcgacga-gattctcacccg-3′ for RM-CK E309D (Kelly et al., 2005). The constructs were transformed into *E. coli* BL21(DE3) and cultured for protein production as described previously (Wu et al., 2008). M1-CK was constructed as described previously, and M1-CK N268G was also constructed by bridge PCR with internal primer pairs, 5′-ttcaagaag-cacggccatggattcatg-3′ and 5′-catgaatccatggccgtgcttcttgaa-3′ (Meza et al., 1996). All the constructs were digested with *NdeI* and *XhoI* and ligated into pET 28a. All the clones were verified by sequencing. All the protein products were stocked with 50% glycerol at −30 °C. Concentration of protein was determined by Commassie Plus protein assay reagent (BioRad).

2.2. Specific activity and thermal inactivation assay

M-CK specific activity assay was based on the method described by Hughes and modified to fit different assay conditions (Hughes, 1962). The basal reaction buffer of M-CK activity assay was 30 mM Tris, 10 mM $MgCl_2$, and 1 mM DTT (1,4-Dithio-DL-threitol). Assay buffer pHs were adjusted to 7.1, 7.4, 7.7 or 8.0 at either 40 °C, 35 °C, 30 °C, 25 °C, 20 °C, 15 °C, 10 °C or 5 °C, and with 12.5 mM PCr, 1 mM ADP and 0.0125 ng enzymes in reaction solution of 80 μL (Wu et al., 2008). After 15 min, the reaction was terminated with *p*-hydroxyl-mercuribenzoate (50 mM, 10 μL), then α-naphthol (2%, w/v; 50 μL) and diacetyl (1:200, v/v; 50 μL) were added for the colorimetric reaction and spectrophotometric absorption was measured at 520 nm. Creatine produced in each reaction was quantified using a creatine standard curve, and specific activity U was defined as 1 μmole of creatine formed per min per mg of enzyme.

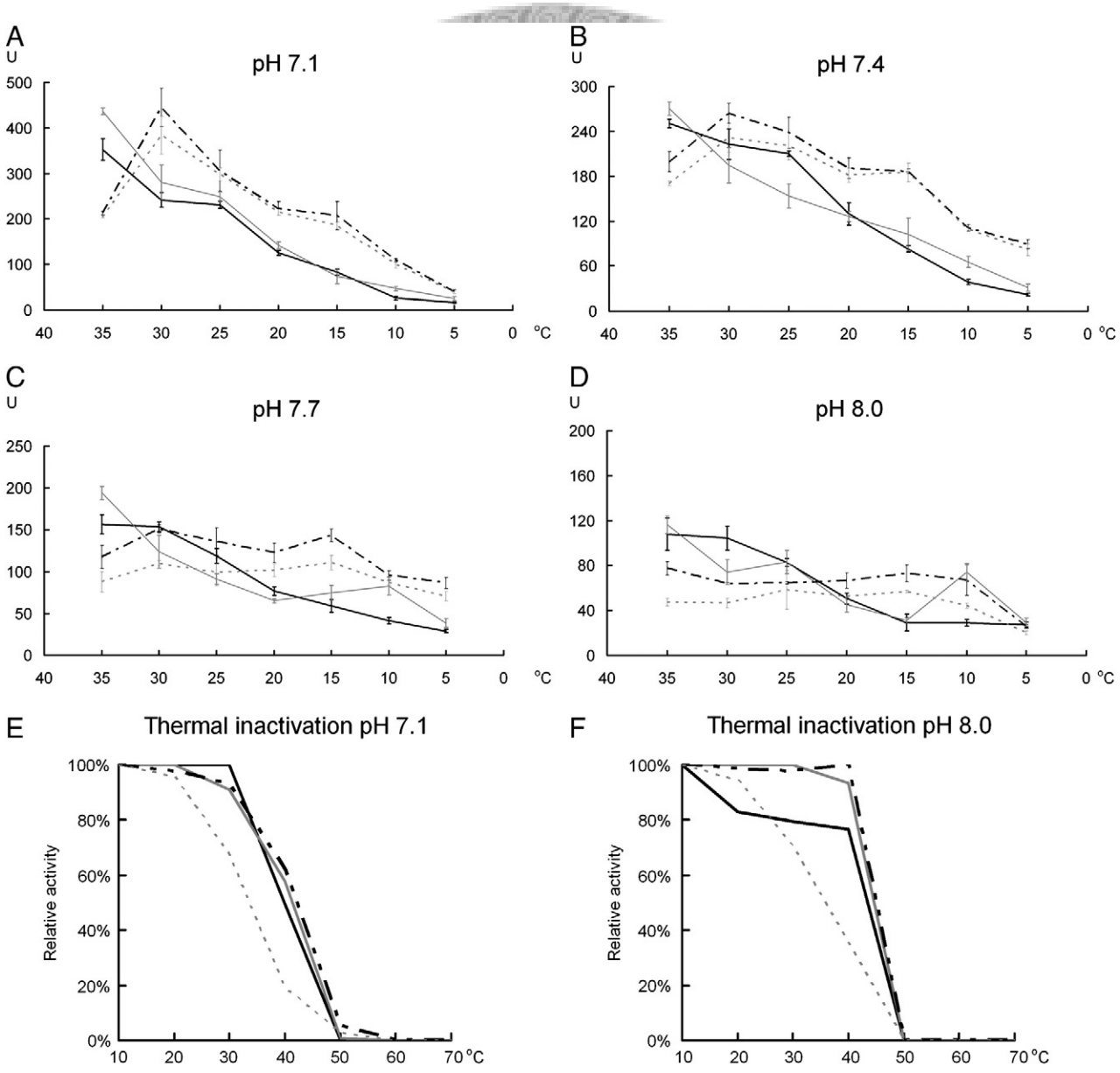

Fig. 2. Specific activities and thermal inactivation curves of RM-CK, RM-CK G268N, M1-CK and M1-CK N268G at different pH and temperature. Specific activities of the two wild-type and mutant M-CKs at (A) pH 7.1, (B) pH 7.4, (C) pH 7.7, (D) pH 8.0, and at temperature from 35 to 5 °C. Thermal inactivation curves at (E) pH 7.1 and (F) pH 8.0. ▬▬: RM-CK, ▬▬: RM-CK G268N, ▬ ▬: M1-CK and ·····: M1-CK N268G. 1 U is 1 μmole of creatine formed per min per mg of enzyme. Vertical bars are standard deviations (SD). Thermal inactivation curves are normalized with the highest specific activity of enzymes of 10 °C heat treatment, ($n>3$, $P<0.05$).

For the thermal stability assay, enzymes were diluted with assay buffer (30 mM Tris, 10 mM $MgCl_2$, and 1 mM DTT, pH 7.1 or 8.0) to 0.0025 mg/mL in a 100 μΛ solution, then incubated at either 10 °C, 20 °C, 30 °C, 40 °C, 50 °C, 60 °C or 70 °C for 30 min. After this heat treatment, enzymes were assayed at 35 °C.

2.3. Kinetic assay and data analysis

Kinetic analyses of CKs were carried out as described in Cleland (1979) (Cleland, 1979). The enzyme specific activity assays for biochemical kinetic analysis of CKs were carried out only at pH 7.1 and 8.0, and at either 35 °C, 25 °C or 15 °C. ADP concentration range was from 0.1 to 2 mM and PCr was at 120 mM when determining the K_m^{ADP}. PCr concentration range was from 0.5 to 120 mM and ADP was at 2 mM when determining the K_m^{PCr}. In each reaction, 6 ng of enzyme was added, and the reaction time was 7 min for the 35 °C reaction and 15 min for the 25 °C and 15 °C reactions with volume of 40 μL each. k_{cat}, K_m^{ADP} and K_m^{PCr} were calculated using the double reciprocal plot of Michaelis–Menten equation. All the reactions were repeated at least 3 times and with more than 1 batch of recombinant proteins. Molecular weights were calculated using the ExPASy Proteomics Server website (Swiss Institute of Bioinformatics), and concentrations of enzymes were calculated using these data.

Using the data processing methods described in Cleland (Cleland, 1979), the pH profiles that showed a decrease in log V_{max}/K_m as pHs were decreased were fitted to Eq. (1),

$$\text{Log}\ (V_{max}/K_m) = \log\ [C/(1+H/K_1)], \quad (1)$$

while the pH profiles that showed a decrease in log V_{max}/K_m as pHs were increased were fitted to Eq. (2),

$$\text{Log}\ (V_{max}/K_m) = \log\ [C/(1+K_1/H)]. \quad (2)$$

C is the pH independent value of V_{max}/K_m, H is the concentration of hydrogen ions and K_1 represents the dissociation constant of a specific group of the enzyme (Wang et al., 2006). In this study the resulting K_1 values were the apparent pK_a of the thiol group of cys^{283} of RM-CK, M1-CK and their mutants at different temperatures.

2.4. Circular dichroism

Circular dichroism (CD) spectra were recorded on a Jasco J715 spectropolarimeter at temperatures 35 °C, 25 °C, 15 °C or 5 °C (Kelly et al., 2005). For far-UV spectrum, a 1 mm path length quartz cuvette of 200 μL was used at a scan speed of 20 nm min^{-1} from 200 nm to 250 nm. The solution for CD contained 2.32 μM of M-CK protein in

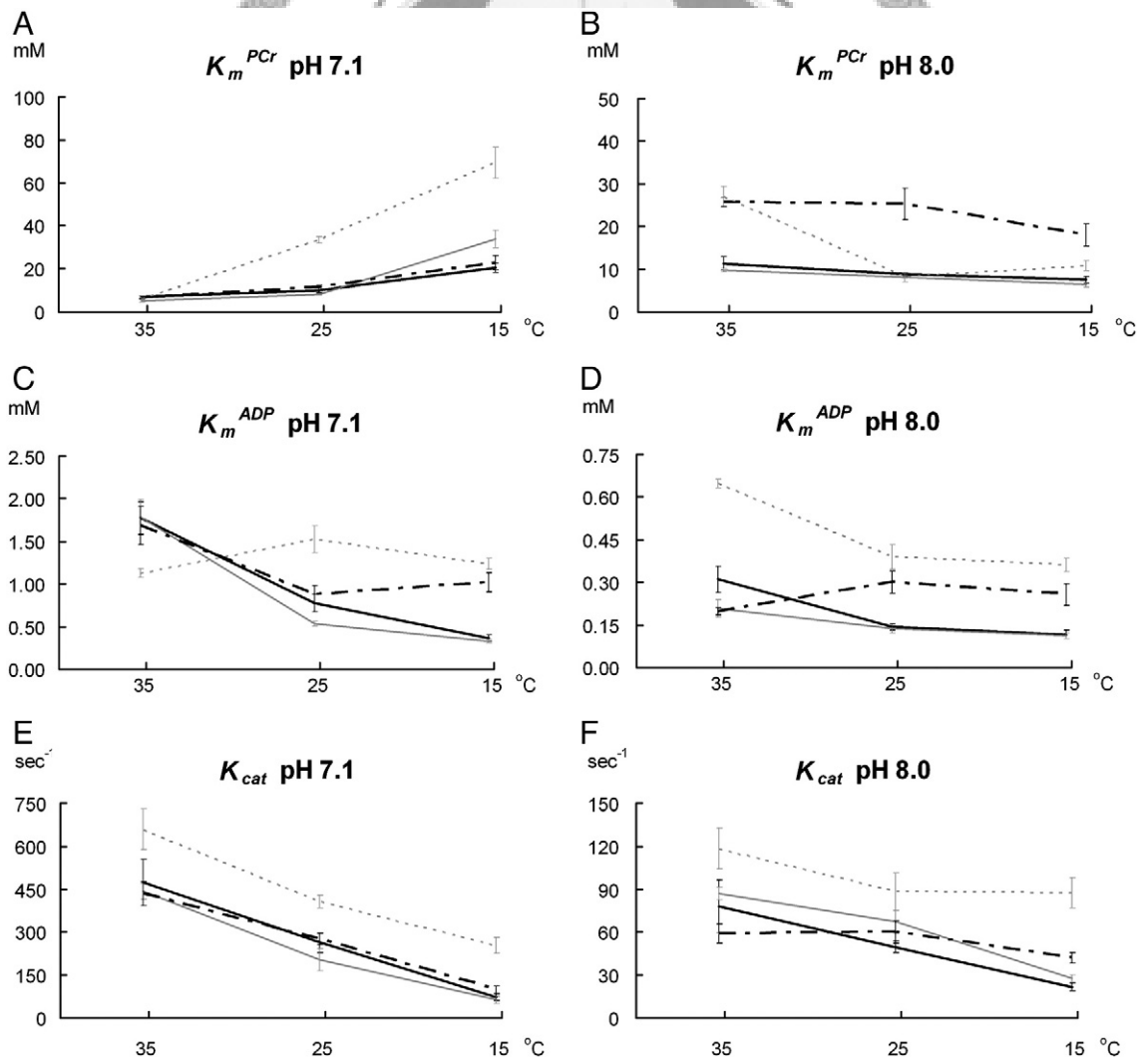

Fig. 3. Kinetic analyses of RM-CK, M1-CK and their mutants at different pH and temperature. (A) K_m^{PCr}, at pH 7.1; (B) K_m^{PCr}, at pH 8.0; (C) K_m^{ADP}, at pH 7.1; (D) K_m^{ADP}, at pH 8.0. (E) k_{cat}, at pH 7.1; (F) k_{cat}, at pH 8.0 of RM-CK, M1-CK and their mutants. ▬▬: RM-CK, ▬▬: RM-CK G268N, ▬ ▬: M1-CK and ·····: M1-CK N268G, ($n>3$, $P<0.05$).

activity assay buffer at pH 7.1 or 8.0, in the absence of ADP and PCr. Data were collected per 0.1 nm and an average of 8 spectra were corrected by subtraction of spectra recorded on the activity assay buffer in the absence of enzyme. Raw data were smoothed by moving average of 19 nearby data.

2.5. Crystallization and X-ray diffraction data collection

The hanging-drop vapor-diffusion method at 25 °C was used for initial crystallization screening of RM-CK G268N and M1-CK. RM-CK G268N crystals grew reproducibly with 10 mg/mL protein in storage solution (30 mM Tris-base, 10 mM $MgCl_2$, 1 mM DTT, pH 7.1, 1 μL) and reservoir solution (20% PEG 3350, 190 mM tripotassium citrate, 0.5 μL), and extended using a Silver Bullets Bio kit (Hampton Research) (0.16% Glycyl-glycyl-glycyl-glycine, 0.16% L-Leucyl-glycyl-glycine, 0.16% Met-Ala-Ser, 0.16% Glycyl-glycyl-glycyl-glycyl-glycine, 0.16% Ala-Leu, 0.16% Tyr-Leu, 20 mM HEPES, pH 6.8, 0.5 μl) (Yeh et al., 2007). The RM-CK G268N crystals grew as hexagonal pillars with dimensions of 0.3 mm × 0.3 mm × 0.8 mm. The crystals were cryoprotected in liquid nitrogen by quick transfer from the hanging drop before data collection. All data sets were taken on a Rigaku R-AXIS IV++ imaging plate detector system using double-mirror-focused CuKα X-ray radiation generated from a Rigaku RU-300 rotating anode operating at 50 kV and 80 mA. Data were indexed, integrated, and scaled using HKL 2000 software packages (Liu et al., 2004).

2.6. Structure determination and refinement

The initial phases of RM-CK G268N were solved by using the molecular replacement software MOLREP of the Collaborative Computational Project, Number 4 (CCP4) suite using a truncated PDB (1crk) as search model (Collaborative Computational Project, Number 4, 1994). After initial model refinements by REFMACS (Murshudov et al., 1997), the final model for the RM-CK G268N consisted of 381 amino acid residues from Methionine1 to Lysine381. The R-factor of this model for all reflections was between 127 and 3.3 Å. The R factor of this structure was refined to 19.9%, and the R_{free} value of 26% was obtained by using 5.0% randomly distributed reflections. The Ramachandran plots for both structures did not violate accepted backbone torsion angles. The PDBviewer 4.01 program was used to generate figures. Statistical data are shown in Table 1.

2.7. Substrate substitution

2′-Deoxyadenosine-5′-diphosphate (dADP, 32 mM) and glutathione (reduced form, 8 mM) were dissolved in distilled water as stock solution (Lee et al., 1977). Assay solutions for CK specific activity and kinetic analysis with dADP were as described previously. There were at least 3 repeats in each assay condition and with more than 1 batch of recombinant enzyme products.

2.8. Statistical analysis

Statistical analysis of specific activity and kinetic results are presented as mean ± standard deviation. One-way ANOVA ($P<0.05$) method was used in analysis of differences between assays.

3. Results

3.1. Enzyme activity, thermal stability and kinetics of RM-CK, carp M1-CK and their mutants at different pHs and temperatures

In order to avoid disturbing the overall structure of the RM-CK, only the differences around the important cys^{283} in RM-CK and carp M1-CK were considered (Fig. 1). At the N-terminal of cys^{283}, there are three variations between these two M-CKs: pro^{270}/gly^{270}, gly^{268}/asn^{268} and ala^{267}/his^{267}. All these variations are located outside or at

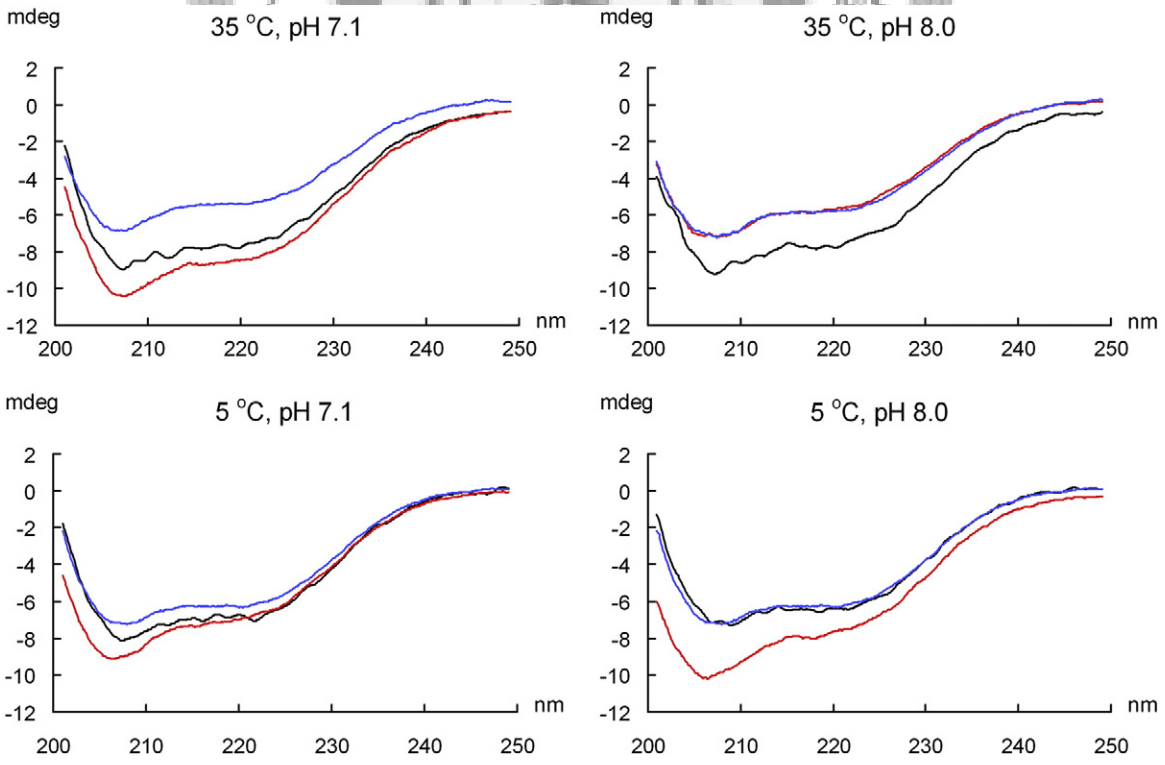

Fig. 4. CD spectra of RM-CK, RM-CK G268N and M1-CK and their mutants at different pH and temperature. CD spectra of RM-CK (red), RM-CK G268N (black) and M1-CK (blue) at pHs 7.1 and 8.0, and at 35 and 5 °C as indicated.

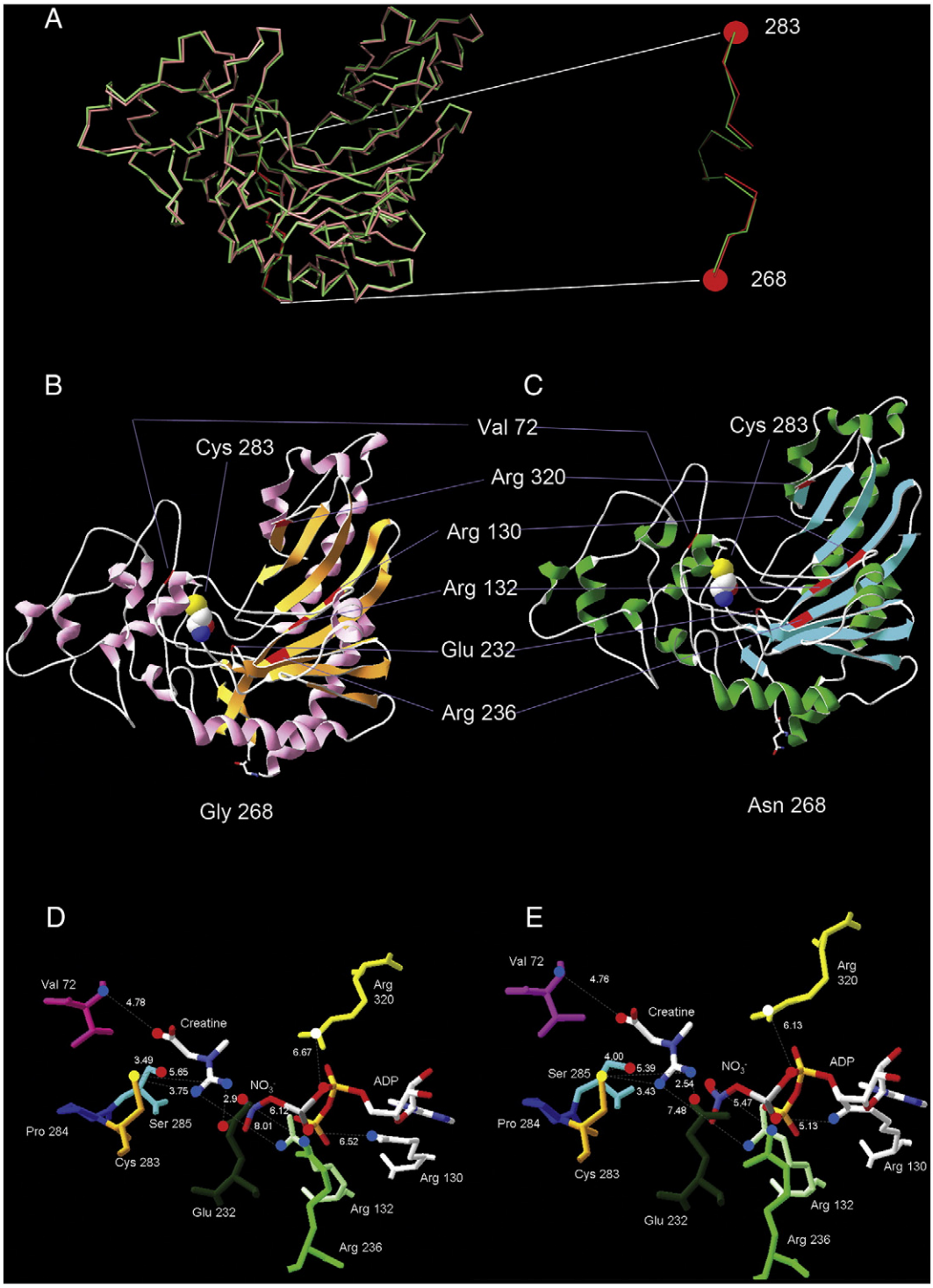

Fig. 5. Crystal structures of RM-CK (2crk) and RM-CK G268N and fine structures of the active sites. (A) The superimposed backbone structures of RM-CK (red) and RM-CK G268N (green). The backbone structures of the two molecules almost fully overlapped with the same conformations. (B) The ribbon models of RM-CK and (C) RM-CK G268N. Cysteine283 are marked as CPK model. The selected residues for calculating the distances between them and the cys^{283} are marked with red and labeled. These models show the positions of the selected residues in their whole protein molecules. The distances between the selected residues of (D) RM-CK and (E) RM-CK G268N to the substrate analog complexes are shown. The residues which would form hydrogen bonds to adapt the intermediate substrate analogs are labeled. The distances of val^{72} N–Cr O; arg^{130} Nη1–ADP O2β; arg^{132} Nη1–Cr Nβ; glu^{232} Oε1–Cr Nα; arg^{236} Nη2–Nitrate O2; cys^{283} S–Cr Nα; and arg^{320} Nη1-ADP O1α are presented in Å. Distances between the selected residues are shorter in RM-CK G268N. All the models were processed using the Swiss-model PDB viewer 4.01.

the edge of any secondary structures. Thus, they were chosen as the objects of this study.

The three RM-CK mutants showed different pH-temperature specific activity patterns compared to wild-type RM-CK (Fig. 2, and Fig. A of online Supplemental materials) (Wu et al., 2008). At pH 7.1, all three RM-CK mutants showed higher specific activities than the wild-type at 35 °C, with RM-CK G268N 23 ± 2%, and A267H and P270G more than 30 ± 7% higher than the wild-type. This is the first time that substitution of a single residue in M-CK has shown the enzyme to gain higher specific activity than its wild-type. At pH 7.7

Table 2
Distances between residues surrounding the active site.

(a)

	RM-CK G268N Cα distances					
	Val^{72}	Arg^{132}	Glu^{232}	Arg^{236}	Arg^{292}	Arg^{341}
Val^{72}		20.10	14.86	19.41	17.16	13.96
Arg^{132}	20.40		13.39	7.44	5.48	11.33
Glu^{232}	14.86	13.42		7.84	15.10	16.83
Arg^{236}	19.69	7.29	8.00		11.48	14.90
Arg^{292}	17.57	5.38	15.12	11.30		7.43
Arg^{341}	14.10	11.95	16.99	15.25	8.13	

(b)

	RM-CK Cα distances		
	RM-CK (2crk)	RM-CK G268N	D
Val^{72}	5.42	5.65	−0.23
Arg^{132}	15.99	15.41	**0.57**
Glu^{232}	9.77	9.66	**0.11**
Arg^{236}	14.39	13.84	**0.54**
Arg^{292}	14.23	13.61	**0.62**
Arg^{341}	12.03	11.61	**0.42**

a. Distances between each Cα of residue pairs were calculated using the model of RM-CK (2crk) and RM-CK G268N in the Swiss-PDB viewer. Unit is in Å. Numbers in bold font in the RM-CK G268N represent shorter distances comparing to the same residue pairs of RM-CK (2crk). b. Differences in distances of selected residues Cα to the thiol group of cys^{283}. D are their differences.

and pH 8.0 at 10 °C; however, only RM-CK G268N exhibited 2 to 2.5-fold higher specific activity than the wild-type, comparable to *C. carpio* M1-CK (Fig. 2A-D) (Wu et al., 2008). While specific activity pH-temperature patterns of carp M1-CK N268G mutant were similar to the wild-type carp M1-CK from pH 7.1 to pH 7.7, at pH 8.0, its specific activity was 50% lower than both wild-type carp M1-CK and RM-CK G268N. Since RM-CK A267H and RM-CK P270G showed no cold-adapted characteristics, their kinetics and structures were not further studied.

Thermal inactivation assay of the M-CKs showed that wild-type RM-CK, RM-CK G268N and carp M1-CK were inactivated at the same temperature range around 40 °C at pH 7.1; however, the inactivation temperature of M1-CK N268G at pH 7.1 was below 40 °C (Fig. 2, E–F). At pH 8.0, the thermal stability of M1-CK and RM-CK G268N actually improved, whereas wild-type RM-CK and wild-type M1-CK N268G started to lose their activity when the 30 min pre-assay heat treatment temperature was above 20 °C. These results suggest that N268 contributes significantly to the thermal stability of M1-CK and RM-CK G268N.

Both K_m^{Pcr} and K_m^{ADP} values of RM-CK G268N were similar to those of wild-type RM-CK at pH 7.1 and pH 8.0 and from 35 °C to 15 °C (Fig. 3A–D). However, both K_m^{Pcr} and K_m^{ADP} of M1-CK N268G fluctuated widely in comparison with those of M1-CK, RM-CK and RM-CK G268N. The k_{cat} values of RM-CK G268N were also similar to those of the wild-type (Fig. 3E–F).

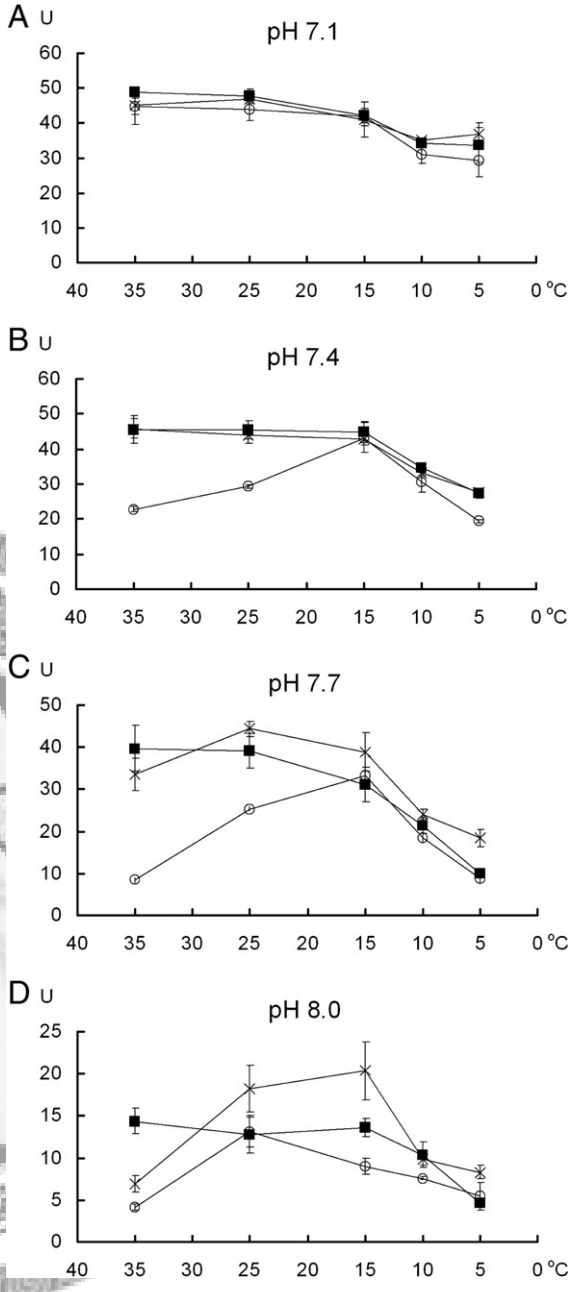

Fig. 6. Specific activities RM-CK, M1-CK and their mutants with dADP substituting ADP as substrate. Specific activities with dADP substitution at different temperature and pH. (A) pH 7.1; (B) pH 7.4; (C) pH 7.7; (D) pH 8.0. —o—: RM-CK; —■—: RM-CK G268N; —×—: carp M1-CK. 1 U is 1 μmole of creatine formed per min per mg of enzyme. Vertical bars are standard deviations (SD), (n>3, P<0.05).

3.2. Secondary and crystal structures of RM-CK G268N

At 35 °C, and at both pH 7.1 and pH 8.0, there were differences in the CD spectra between RM-CK G268N and the two wild-types (Fig. 4). At 5 °C, and especially at pH 8.0, however, the CD spectra of the RM-CK G268N mutant and wild-type M1-CK were almost identical, suggesting that a mutation of G268N could cause the RM-CK mutant to adopt similar overall secondary structures as carp M1-CK at low temperatures.

Unfortunately, attempts to grow crystals of M1-CK consistently failed, thus only RM-CK G268N crystals were available for structural studies. The crystal structure of RM-CK G268N solved by molecular replacement using RM-CK as model and refined to 3.3 Å resolution, consisted of two independent RM-CK G268N dimers contained in an asymmetric crystal unit (Table 1). The backbone structure of RM-CK G268N was essentially identical to the RM-CK (PDB accession code: 2crk) (Fig. 5A) (Rao et al., 1998). The Cα group of cys^{283} in RM-CK G268N was 0.2 Å shorter than in wild-type RM-CK, and the secondary structures of RM-CK G268N were identical to wild-type RM-CK (Fig. 5B–C) (Rao et al., 1998). Asn^{268} is located at the periphery of the molecule with the side chain pointing outward. The model structure of RM-CK was consistent with previous CK structure studies in which val^{72}, arg^{130}, arg^{132}, glu^{232}, arg^{236} and arg^{320} set the boundary of the active site (Uda et al., 2009; Jourden et al., 2005; Edmiston et al., 2001). Distances between these 6 residues were calculated to show the 3-D structure of the active site pocket (Table 2) (Uda et al., 2009). The distances between the creatine binding residues (val^{72} and glu^{232}) and the nucleotide binding residues (arg^{132}, arg^{236}, arg^{292} and

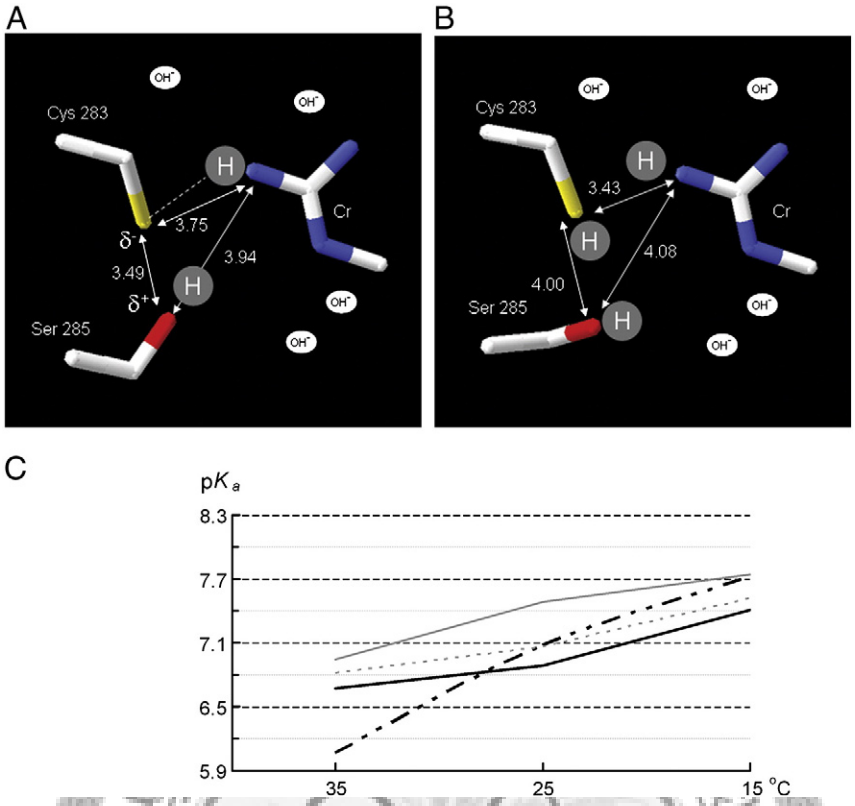

Fig. 7. The atom positions of cys[283], ser[285] and Cr amine group of RM-CK and RM-CK G268N, and *pKa* values of RM-CK, M1-CK and their mutants derived from kinetic results. Distances of atom positions of the thiol of cys[283], hydroxyl of ser[285] and the amine group of Cr are shown in (A) RM-CK (2crk) and (B) RM-CK G268N. At pH 7.7 or above the thiol group of cys[283] in RM-CK is presented to be deprotonated, while that of RM-CK G268N is protonated. Red atom is oxygen; blue is nitrogen and yellow is sulfur. Distances between atoms are presented in Å. These models were processed by the Swiss-model PDB viewer 4.01. (C) *pKa* of RM-CK, M1-CK and mutants. ▬▬: RM-CK, ▬▬: RM-CK G268N, ▬ ▪ ▬: M1-CK and ·····: M1-CK N268G.

arg[320]) were shorter in RM-CK G268N than in wild-type RM-CK. The distances from val[72] to arg[292] and glu[232] to arg[236] were shorter by 0.4 Å and 0.2 Å, respectively, in RM-CK G268N than in wild-type RM-CK (Fig. 5D).

3.3. Substrate substitution of CKs

To test whether the active site of the RM-CK G268N mutant was smaller and thus likely to favor catalysis with a smaller substrate, ADP was replaced by dADP (Fig. 6) (Lee et al., 1977). At pH 7.1, specific activities of RM-CK, M1-CK and RM-CK G268N were similar from 5 °C to 35 °C. At pH 7.4 and pH 7.7, M1-CK and RM-CK G268N were consistently and significantly more active than RM-CK at 35 °C, but activity of RM-CK increased as temperature decreased and reached a maximum at 15 °C. At pH 8.0, activity of the RM-CK G268N changed a little and was higher than the wild-type through 35 °C to 10 °C though not significantly except at 35 °C. At this pH, at 35 °C, M1-CK activity was low as RM-CK, but increased significantly as temperature decreased and reached a maximum at 15 °C. These results are consistent with the suggestion that the mutant possessed a smaller active site, and that M1-CK has evolved to possess changeable conformations that can adapt to the synchronized changes in body temperature and intracellular pH of carp (Albers et al., 1983).

4. Discussion

The temperature of the habitat of the common carp varies from 5 °C to >35 °C and its extracellular and intracellular fluid pH varies inversely with body temperature (Hwang and Lin, 2002; Stecyk and Farrell, 2006; Yancey and Somero, 1978). Previously, it was suggested that a 16 °C range of temperature cannot be executed by one metabolic profile (Guderley, 1990). However, a more recent analysis of the three carp muscle-specific creatine kinase isoforms revealed that only M1-CK functions well at pHs from 7 to 8 and from 0 °C to 35 °C (Wu et al., 2008).

Biochemical analyses indicate that the K_m and k_{cat} of psychrophilic enzymes from Antarctic and Arctic organisms are higher than those of their mesophilic or thermophilic homologous due to lower activation energy (E_a) (Marshall, 1997). The molecular expectation of enzyme function at low temperature has been examined by comparing homologous enzymes from organisms whose habitats span a wide range of temperatures and it was found that the thermal denaturation temperature of cold-adapted enzymes is lower than that in mesophilic homologous which suggests higher flexibility in cold-adapted enzymes (Feller and Gerday, 1997; Hoyoux et al., 2004; Smala's et al., 2000; Bae and Phillips 2004).

High flexibility is accompanied by a trade-off in stability, resulting in heat lability, and in the few cases studied, cold lability (Siddiqui and Cavicchioli, 2006). For RM-CK, a single mutation in D54G decreased the midpoint temperature of thermal inactivation by 16 °C and resulted in a 79% decrease in its activity at 25 °C, inferring that increase in flexibility of the protein in turn caused partial unfolding of the protein (Feng et al., 2007). However, in the present study, CD spectra, specific activity and thermal stability showed that M1-CK and RM-CK G286N are both flexible and yet thermally stable and function from pH 7 to pH 8 and from 35 °C to 10 °C. The residue change in the RM-CK mutant is located at the surface of the molecule, thus intracellular pH change may be an important factor affecting flexibility of the protein. These kinetic properties suggest that in addition to flexibility there may be other possible factors allowing

M1-CK and RM-CK G286N to function over a broad range of temperatures. The crystal structure of RM-CK G268N reveals one such possible factor.

RM-CK has been cloned and its crystal structure, 2crk, has been resolved (Rao et al., 1998; Putney et al., 1984). The cys^{283} residue is important in keeping creatine anchored and positioned for nucleophilic attack on the γ-phosphorus of MgATP within a SN2 type reaction (Lahiri et al., 2002; Milner-White and Watts, 1971). Arg^{96}, arg^{132} (creatine binding in Fig. D), arg^{236}, arg^{292} and arg^{341} are correlated to nucleotide binding, and asp^{232}, glu^{232}, val^{72} and asp^{326} are correlated to creatine binding (Fig. B of online materials) (Uda et al., 2009; Wood et al., 1998; Tanaka and Suzuki, 2004; Cantwell et al., 2001; Feng et al., 2007). The crystal structure of RM-CK G268N shows that the distances of these residues to the target atoms of the substrates are all shorter in the mutant than in the wild-type, and thus may improve stabilization of the substrates docking in the active site in the mutant. Further modeling shows that the thiol of cys^{283} and the hydroxyl of ser^{285} of RM-CK G268N moved 0.44 and 0.77 Å, respectively, away from the active site. The distance between the thiol of cys^{283} and the hydroxyl of ser^{285} lengthened by 0.51 Å in RM-CK G268N (Fig. 7A–B).

In RM-CK, it has been shown that ser^{285} and pro^{284} decrease the pK_a of cys^{283} thiol by around 2.2 pH units, thus, at neutral pH, cys^{283}-anion is important in maintaining the linear alignment necessary for associative inline transfer of a phosphoryl group (Wang et al., 2006, 2001). In the present work, the apparent pK_a of RM-CK, RM-CK G268N and M1-CK at pH 7.1 and pH 8.0 and from 35 °C to 15 °C were calculated based on the kinetic data, and pK_a values of RM-CK G268N were higher than those of RM-CK (Fig. 7C) (He et al., 2007). The most striking feature is that at 15 °C, the pK_a values of RM-CK G268N and M1-CK are almost identical at pH 7.7, as well as 0.35 pH units higher than that of RM-CK. In the case of carp M1-CK, as ambient temperature decreased and pH of intracellular fluid increased above pH 7.7, deprotonation of cys^{283} thiol became less likely. At pH 8.0, the kinetic mechanism appeared rapid equilibrium random in both directions on the basis of both initial velocity studies (Morrison and James, 1965; Cook et al., 1981).

According to Cook et al. (1981), in substrate phosphocreatine binding, the rate constants k_8/k_5 ratios are 2.5 at 25 °C and 9.3 at 12 °C, k_8 being the rate constant of phosphocreatine release from E-MgADP-phosphocreatine and k_5 being that of proton release to H_2O from EH-MgADP-phosphocreatine, and k_5 appears to be highly temperature sensitive (Cook et al., 1981). In the reverse reaction, the rate constants k_3/k_2 ratios are 4.0 at 25 °C and 11.3 at 12 °C, k_3 being the rate constant of product creatine release from EH-MgADP-phosphocreatine, and k_2 that of phosphocreatine release from EH-MgADP-phosphocreatine, and k_2 is more temperature dependent than k_3. Thus, at low temperature, phosphocreatine and proton are stickier, and if k_3 and k_8 are less temperature sensitive and change little, k_2 and k_5 should decrease. The outcome, then, also would favor the release of phosphocreatine or creatine from the active site (Cook et al., 1981).

Assay with a smaller substrate, dADP, also showed that activities of RM-CK G268N from pH 7.4 to pH 8.0 are all higher than the wild-type though not as prominent as M1-CK. And the K_m^{dADP} of RM-CK G268N are all smaller than the wild-type in all conditions (data not shown). Thus one change in G268N in RM-CK indirectly causes a tiny decrease in the 3-D structures of the active site of RM-CK G268N, and this decrease also increases the apparent pK_a of the RM-CK G268N. Although M1-CK still functions better from pH 7.1 to pH 8.0 and 10 °C to 30 °C due to G268N as well as other changes in amino acid residues, it may still be concluded that one change in G268N at a non-critical part of the enzyme may make a significant contribution in causing RM-CK G268N and M1-CK to be more cold-adapted. In the future, attempts will be made to change multiple sites such as G268N and A267H to see if CK enzymes can be made to function well across pHs 7 to 8 and from 35 °C to 5 °C; their 3-D structures will be studied. At the same time, the physical chemical mechanism of the residues affecting the active site of CK should be clarified, too.

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