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鯉魚肌肉型肌酸激酶在低溫下的活性取決於具極性支鏈的第

268位置胺基酸

Activity of the common carp muscle form creatine kinase at low
temperature depends on residue 268 with polar side chain

林希潔 Hsi-Chieh Lin

指導教授: 嚴宏洋博士 Hong-Young Yan, Ph.D.

許祖法博士 Cho-Fat Hui, Ph.D.

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摘要

鯉魚屬於廣溫性魚類, 在 35 到 5°C 的環境中依然能維持正常的生理與代謝功能。對於鯉魚來說, 如何在此廣泛的溫度下維持細胞的能量是很重要的課題。肌酸激酶是一個很重要的能量平衡酵素, 可以調控並維持細胞內的能量平衡。肌肉型肌酸激酶可提供 ATP 給肌肉細胞以進行收縮。鯉魚具有 3 種同功異構的肌肉型肌酸激酶(M1-, M2- 和 M3-CK), 其中只有 M1-CK 能夠在低溫下維持活性, 而恆溫動物兔子的肌肉型肌酸激酶 (RM-CK) 在低溫下則會失去活性。M1-CK 和 RM-CK 的胺基酸序列有 86% 的相似性, 比較兩者胺基酸的差異, 以定點突變技術將 RM-CK 第 268 位置的 glycine 突變成 asparagin, 稱為 RM-CK G268N。RM-CK G268N 在 10°C pH 8.0 的情況下, 活性是 RM-CK 的 2.5 倍。從這個實驗結果我們推論 asparagin 268 或許是 M1-CK 能在低溫下作用的其中一個重要原因。因此我們利用定點突變技術將 M1-CK 和 RM-CK 第 268 位置的胺基酸突變成 aspartic acid, lysine 和 leucine, 去研究是否是因為極性的支鏈影響其在低溫下的功能。從活性的結果來看, 當 268 的胺基酸含有極性的支鏈時, 其在低溫下的活性會比非極性的突變高, 顯示極性的支鏈的確會影響 M-CK 低溫下的活性。從 CD 的實驗確認 268 胺基酸的改變不會影響整體的二級結構。熱不穩定性實驗則顯示含有極性 268 胺基酸的 CK 在結構上呈現比較穩定的狀態。在低溫時, 水分子和水分子容易形成鍵結, 造成酵素表面上的分子間親水性作用力下降, 而酵素分子內的疏水性作用也降低, 使得酵素的結構處於較不穩定的狀態。從結構上來看, 當 268 胺基酸為親水性時, 會和附近的其他親水性胺基酸在蛋白質表面形成一個連續的親水性區域, 這個親水性區域可以增加對水分子吸引力, 使水分子和親水性胺基酸形成氫鍵, 增加表面的親水性作用及分子內的疏水性作用以穩定 CK 的結構, 因此我們認為 M1-CK 藉由 268 位置的極性胺基酸來增加和水分子之間的鍵結來穩定結構, 使其可在低溫下維持活性。

Abstract

The common carp (*Cyprinus carpio*) required to maintain physiologic and metabolic capabilities when temperature of their habitat varies over a large temperature range 5 °C to 35 °C. At this broad range of temperature, ATP production in energy demanding cells is an important criterion to maintain the common carp. Creatine kinase is an important energy homeostasis enzyme. Muscle-specific creatine kinase (M-CK) catalyses the reversible reaction that converts phosphocreatine and ADP to creatine and ATP and thus provides ATP for muscle contraction. Previously, three common carp CK isoforms (M1-, M2- and M3-CK) were cloned. M1-CK has been suggested to have evolved to become the only CK that functions over the ranges of intracellular pH and body temperature variation that occurs in the common carp. The primary structures of RM-CK (M-CK of homeothermal rabbit) and carp M1-CK share 86% identity. By comparing the differences between RM-CK and M1-CK, and with RM-CK cDNA as template, RM-CK G268N show higher specific activity than wild type at pH 8.0 at 10 °C. This result suggests N268 residue plays an important role in conferring the appropriate 3D structure to M1-CK to function at low temperature. Therefore, we mutated 268 residues to aspartic acid, lysine or leucine and examined their properties.

The mutants with polar side chain at residue 268 had higher activity and could maintain their function at low temperature. From the thermal stability, the polar mutants were more stable than non-polar mutants. Examining the structure surface of these mutants, when residue 268 was hydrophilic, it could connect nearby hydrophilic residues to form continuous hydrophilic region. This hydrophilic region could hydrogen bond with water, which then stabilizes the enzyme and results in maintaining activity at low temperature.



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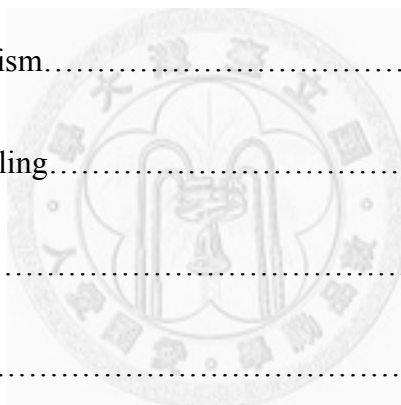


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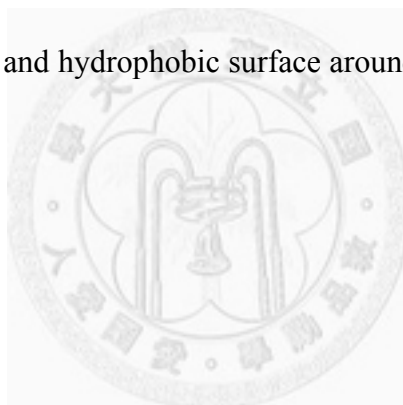


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Abbreviations

ADP: adenosine diphosphate

ATP: adenosine triphosphate

Cr: creatine

PCr: phosphocreatine

DTT: 1, 4-dithiothreitol

LB: Luria-Bertani medium

CK: creatine kinase

M1-CK: M1 subisoform of muscle specific creatine kinase of common carp

RM-CK: muscle form creatine kinase of rabbit

TSAC: transition state analog complex, including creatine kinase, creatine, Mg-ADP and nitrate

CD : circular dichroism spectroscopy

RM-CK mutants: RM-CK G268N, RM-CK G268D, RM-CK G268K and RM-CK G268L

M1-CK mutants: M1-CK N268G, M1-CK N268D, M1-CK N268K and M1-CK N268L

Polar mutants: RM-CK G268N, RM-CK G268D, RM-CK G268K, M1-CK, M1-CK

N268D and M1-CK N268K

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Introduction

Influence of water temperature on fish, and common carp has a well-developed capacity to modify muscle properties in response to low temperature

Water temperature is a major factor that influences fish physiology. In fish, heat generated by metabolism is carried via blood to the gills, where it is lost to the environment (Stevens and Sutterlin, 1976). Consequently, body temperatures of fish depart little from ambient water temperature (Carey, 1973; Reynolds et al., 1976).

Cultivating fishes are impacted when ambient water temperature change. Cold stress is a severe challenge to aquaculture industry in many countries including Taiwan. There is a big loss in aquaculture from the drastic drop of water temperature to below 15°C during winter (Johnston and Temple, 2002). The cold damages aquaculture fishes, including tilapia (*Oreochromis mossambicus*), milkfish (*Chanos chanos*), grouper (*Epinephelus coioides*) and cobia (*Rachycentron canadum*). When surrounding temperature goes below 15°C, these species lose their swimming ability and become imbalance, and finally die (Johnston and Temple, 2002). This cold-induced economic fishery losses in Taiwan amount up to US \$ 10 million annually. Similarly in southern United States and Israel, water temperature during winter may drop to levels that cause severe growth inhibition and mortality to aquaculture tropical fishes (Snodgrass, 1991).

Variations in body temperature impact fish physiology in two ways: (1) temperature determines the rate of chemical reactions, and even modest reductions in temperature markedly depress reaction rates; (2) temperature dictates the point of equilibrium between the formation and disruption of non-covalent forces which stabilize both macromolecular and molecular structures of physiological importance, and the binding of ligands to proteins (Wood, 1993).

Structural flexibility is necessary for both protein function and well-being of biological membranes. But cold temperature can constrain this flexibility, thereby stabilizing less active conformations. Conversely, high temperatures promote conformational plasticity that biological structure can no longer be maintained (Ciardiello et al., 2000).

The significant changes in body temperature bring a serious challenge to the maintenance of integrated physiological function. Among fishes, this challenge is met and the effects of acute temperature change obviated in various ways: (1) some fish (endothermy) have evolved specialized mechanisms for the production and retention of metabolic heat: (2) most fish are essentially isothermal with their environment and cope with the problems of poikilothermy (i.e., variable body temperatures) by a diversity of mechanisms (Rome et al., 1985).

Fish deal with thermal stress by a variety of mechanisms, including: behavioral

adjustments, physiological responses to temperature change (shock, within one day), and acclimatory or adaptational adjustments to persistently altered temperatures (acclimation, during weeks to months or adaptation, a much longer period) (Bernal et al., 2001; Wood, 1993).

Although many fish can thermoregulate behaviorally, microhabitat selection is not effective in dealing with long-term fluctuations in environmental temperature. Consequently, fish possess a variety of alternate mechanisms for coping with the extremes of environmental temperature (Wood, 1993).

In thermal acclimation, thermal compensation is more evident in eurythermal fishes that must acclimatize to seasonal or daily fluctuations in temperature. Individual fish can survive because of their physiology and biochemistry is altered in response to temperature change (Gamperl et al., 2002). Such environmentally induced phenotypic plasticity generally requires a period of adjustment that may last from days to weeks. Acclimation to temperature extremes both improves performance at the acclimation temperature and extends the range over which normal activity can be maintained.

In thermal adaptation, polar teleosts exhibit higher rates of metabolism than their tropical counterpart, they display higher rates of oxygen consumption when acclimated to cold than warm temperatures (Kanungo and Prosser, 1959). At the cellular level, the capacity for ATP production by aerobic means is commonly elevated at cold

temperatures (Hazel and Prosser, 1974). Although pathways of carbohydrate (Stone and Sidell, 1981), lipid (Sellner and Hazel, 1982), RNA/DNA (Das, 1967), and protein metabolism all acclimate to temperature change, the nature of these compensations is frequently highly specific. Thus, temperature acclimation involves a total reorganization of cellular metabolism rather than a simple up- or down regulation of general metabolic rates. The adaptive significance of acclamatory adjustments in membrane fluidity must be judged by temperature compensated on membrane function. Modulation of fluidity has also been implicated in the elevated activities of succinate dehydrogenase (Hazel, 1972), cytochrome oxidase (Wodtke, 1981), and Na⁺/K⁺-ATPase (Raynard and Cossins, 1991) typical of cold-acclimated fish, as well as the compensation of membrane permeability (Hazel and Schuster, 1976) and neural function.

The eurythermic common carp (*Cyprinus carpio*) distributes from Europe to Asia, and lives at temperature range from 10 to 35°C (Hwang and Lin, 2002; Stecyk and Farrell, 2006). They are hardy and tolerant to a wide variety of conditions. It has been known that the pH of extracellular and intracellular fluids of poikilothermic animals varies inversely with body temperature (Yancey and Somero, 1978). In the common carp, blood and intracellular pHs rise 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). Thus, there is interest in

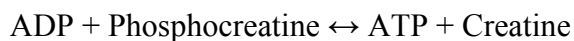
many aspects of their physiological responses such as metabolism (Wodtke, 1981; Guderley, 1990), neuron response (Ven der Linden et al., 2004), endocrine release (Flik et al., 2006) and behavior (Rome et al., 1990; Johnston & Temple, 2002; Guderley, 2004; Ven der Burg et al., 2006). At this broad range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

The common carp can survive well at temperature as low as 10 °C without showing impaired swimming ability (Watabe, 2002). The swimming ability of fish at low temperature was fueled by muscle contraction and the efficiency of the acquisition to closely regulate the energy metabolism (Rome, et al., 1985). The common carp has a well-developed capacity to modify muscle properties in response to changes in temperature, which allows it to maintain physiological and mechanical homeostasis under severe thermal stress (Johnston et al., 1990; Wakeling et al., 2000). In most muscle tissues, the ATP regeneration capacity of creatine kinase (CK, EC 2.7.3.2), the key energy metabolic enzyme (Wallimann, 1994), is highly effective, considerably exceeding both ATP utilization as well as ATP replenishment by oxidative phosphorylation and glycolysis (Wallimann et al., 1992). Therefore, we investigate the common carp muscle-form creatine kinase to find out how CK functions in order for eurythermic fishes to adapt to different ambient temperatures.

Creatine kinase is an important energy homeostasis enzyme, and M1-CK subisoform of common carp still functions well at low temperature

Creatine kinase (CK) is a member of the phosphagen (guanidino) kinase family. This family of enzymes is highly conserved and is found throughout the animal kingdom. Other members of the family include arginine kinase (AK), glycoamine kinase (GK), taurocyamine kinase (TK), and lombricine kinase (LK) (McLeish and Kenyon, 2005). Creatine kinase is the only phosphagen kinase found in vertebrates, but it is also found in many invertebrates, including sponges, polychaetes, and echinoderms (Robin, 1964; Watts, 1968, 1971, 1975; Ellington, 2001).

CK catalyzes the reversible transfer of a phosphoryl group from ATP to creatine (Feng et al., 2007).



The most important physiological function of CK is the regeneration of ATP from phosphocreatine (PCr) when high energy is demanded (Wallimann and Hemmer, 1994; Wallimann et al., 1992).

There are four different CK isoforms known in vertebrates; two are found in cytosol, and two are found in mitochondria. The cytosolic forms are called MM-CK (muscle)

and BB-CK (brain), which exist as dimers under physiological conditions (Eppenberger *et al.*, 1967; Wallimann *et al.*, 1992). Under some circumstances, cytosolic CK can exist as the MB heterodimer (Eppenberger *et al.*, 1967; Rosenberg *et al.*, 1981; Quest *et al.*, 1990). 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). The mitochondrial forms are called ubiquitous (Miu-CK) and the sarcomeric (Mis-CK). MiCK also forms dimers, which can readily associate to an octameric form depending on the pH and the concentration of MiCK (Kaldis *et al.*, 1994; McLeish and Kenyon, 2005; Wallimann *et al.*, 1992).

The regulatory function of CK is particularly important in cells that have large and variable energy demands, such as in the brain, heart, muscle, neurons, photoreceptor, retina, spermatozoa and electrocytes. This important regulatory network has come under scrutiny from a number of recent studies suggesting that improper regulation of the CK/PCr system is associated with a variety of disease states, including cardiovascular, neurological, muscle and renal diseases as well as cancer (Schlattner *et al.*, 2006; Wyss and Kaddurah-Daouk, 2000).

MM creatine kinase has different isoforms in different species. In dogs, the hydrolytic cleavage of a basic amino acid, presumably by carboxypeptidase N, is

responsible for conversion of muscle tissue MM1 to MM2 and MM3 (George et al., 1984). In humans, MM-CK isoforms are formed by the successive removal of the COOH-terminal lysine residue from one M subunit at a time, resulting in the conversion of MM-A to isoforms MM-B and MM-C (Billadello et al., 1985). In mammals, there is just one isoform of B-CK, and the two B-CK isoforms of chicken are derived from a single gene by alternative splicing of the second exon (Hossle et al., 1986; Wirz et al., 1990). Additional heterogeneity of B-CK has been shown to be due to alternative initiation of translation or post-translation phosphorylation (Soldati et al., 1990; Quest et al., 1990; Chida et al., 1990; Hemmer et al., 1993).

In teleost fishes, there are four CK isoenzymes termed CK-A to CK-D and are all of cytoplasmic origin. CK-A, CK-C, and CK-D are expressed predominantly in striated muscle, stomach, and testis, respectively, while CK-B is expressed ubiquitously or is confined to neural tissue (Fisher et al., 1978). In trout, a cDNA encoding for a CK named TCK-1 has been demonstrated to show enhanced testicular expression, and an s-CK protein has been purified from sperm (Saudrais et al., 1996). Since *Torpedo* electrocytes have been shown by isoenzyme and two-dimensional electrophoresis to contain the same major CK isoforms as muscle, the two *Torpedo* CKs very likely represent the CK-A isoenzyme (Barrantes et al., 1983; Giraudat et al., 1984; West et al., 1984). Based on a comparison of the tissue specificity of expression of the various

isoenzymes, it has been hypothesized that CK-II of frogs and CK-A of fish correspond to M-CK of mammals and birds, while CK-IV and CK-C correspond to B-CK (Wolff et al., 1985; Mühlebach et al., 1994).

Previously, three common 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 common carp (Sun et al., 2002).

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 common carp M-CK (Tanzer and 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 at temperatures from 5 to 40 ° C, and at pH that resemble the changes in the common carp intracellular pH (7.1–8.0) were studied (Hughes, 1962).

In comparison of the specific activities of M1-, M2- and M3-CK, M1-CK exhibited highest activity, M2-CK, however, was very low at all pH and temperatures, M3-CK was between M1- and M2-CK. M3-CK increased from 30 to 20°C then decreased when below 20° C, yet M1-CK activity remained stable from 30 to 20° C, then increased

significantly at 15° C. At pH 8.0, 15° C, only M1-CK was able to maintain its specific activity at > 50% (156U) its highest specific activity, while specific activity of M3-CK decreased to 25 U. Therefore, it seems that M1-CK can function well in the common carp muscle from 35 to 5° C (Wu et al., 2008).

Comparisons of thermal stability showed that M3-CK was least thermal stable (Wu et al., 2008). A study using 2-D gel on cold acclimation and expression of soluble proteins in the common carp skeletal muscle revealed a downregulation of expression of M2- and M3-CK, and an increased accumulation of their fragments (McLean et al., 2007). M2-CK and M3-CK were both found to be thermal unstable as well as subject to ubiquitination and proteolytic fragmentation at low temperature (McLean et al., 2007). 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 the common carp (Wu et al., 2008).

The difference between RM-CK and M1-CK at low temperature, and residue 268 may play an important role for M1-CK maintains its activity at low temperature.

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. At temperatures < 15 °C and at pH >7.7, specific activities of M1-CK were three to eight-fold higher than specific activities of RM-CK (Wu et al., 2008).

The primary structures of RM-CK and carp M1-CK share 86% identity (Table 1). Well conserved residues have been studied by site directed mutagenesis 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). Arg⁹⁶, arg¹³⁰, arg¹³², arg²³⁶, arg²⁹², arg³²⁰, and arg³⁴¹ have been reported to be transition-state analog complex (TSAC) binding residues, and his⁶¹, his⁹², his¹⁸⁶, and

his²⁹⁵ 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 charged residues, glu²²⁶, glu²²⁷, and asp²²⁸, 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 val⁷¹, asp²³¹ and glu³²⁶, and cys²⁸³ is important but not essential for catalysis (Cantwell et al., 2001; Furter et al., 1993; Forstner et al., 1996; Ohren et al., 2007; Reddy et al., 2000; Tanaka and Suzuki, 2004).

In M-CK, cys²⁸³ 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 (pro²⁸⁴ to ala²⁸⁴), S285A (ser²⁸⁵ to ala²⁸⁵) and C283S/S285C (cys²⁸³ to ser²⁸³/ ser²⁸³ to cys²⁸³) mutants, results suggested that ser²⁸⁵ lowers the pK_a about 1 pH unit and pro²⁸⁴ 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).

By comparing the differences between RM-CK and M1-CK, and with RM-CK cDNA as template, selected site direct mutagenesis were carried out. 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, most mutations were made in the random coil regions and the hinge region between the N- and C-terminals. Three RM-CK mutants, designated as RM-CK A267H (ala²⁶⁷ to his²⁶⁷), RM-CK G268N (gly²⁶⁸ to asn²⁶⁸) and RM-CK P270G (pro²⁷⁰ to gly²⁷⁰), that still retained significant enzyme activity were generated and their enzymatic and physiochemical properties examined (Wu et al., 2011).

RM-CK G268N show higher specific activity than wild type at pH 8.0 at 10 °C. Comparing the amino acid sequences of muscle-form creatine kinase of endothermal animals (mammal) and ectothermal animals (fish), at residue 268, endothermal animals are always glycine and ectothermal animals are always asparagines (Table 2). This result suggests that asn²⁶⁸ residue may plays an important role in conferring a appropriate 3D structure to M1-CK to function at low temperature.

Analysis the crystal structure of RM-CK G268N, the C α group of cys²⁸³ in RM-CK G268N was 0.2 Å shorter than in wild-type RM-CK. The model structure of RM-CK was consistent with previous CK structure studies in which val⁷², arg¹³⁰, arg¹³², glu²³², arg²³⁶ and arg³²⁰ set the boundary of the active site (Uda et al., 2009; Jourden et al., 2005; Edmiston et al., 2001). The distances between the creatine binding residues (val⁷² and glu²³²) and the nucleotide binding residues (arg¹³², arg²³⁶, arg²⁹² and arg³²⁰) were shorter in RM-CK G268N than in wild-type RM-CK. The distances from val⁷² to arg²⁹²

and glu²³² to arg²³⁶ were shorter by 0.4 Å and 0.2 Å, respectively, in RM-CK G268N than in wild-type RMCK. Therefore, a smaller active site might be the reason for improvement in activity at low temperature (Wu et al., 2011).

The difference between glycine and asparagine is the side chain, and the side chain of residue 268 of RM-CK located on the surface of the protein molecule and points out to the solvent. The two polar atom, oxygen and nitrogen of the side chain of asparagine are hydrophilic. A hydrophilic side chains located at molecular surface, where a polar atom can easily form hydrogen bonding with the solvent owing to the flexibility of water molecules (Xu et al., 1997) and hydrophilic side chain may reduce the hydrophobicity on the interface of protein and water (Fersht, 1985; Timasheff, 1993). The water molecular that located at hydrophobic region of the enzyme is at a high energy state because it is at an interface and not in bulk water (Fersht, 1985). Bulk water is where it can fulfill readily all its hydrogen bonding and can stable the molecular that interaction with it (Fersht, 1985).

Cold-induced change in a protein based on the very unusual feature of hydrophobic interaction. At low temperature, protein may be in a cold denatured state, and hydrophobic interaction decreases as a result of the increase in hydration contribution (Privalov, 1990). Polar groups rather than non-polar surfaces promote the formation of hydrogen bonding network at low temperatures (Robinson and Cho, 1999).The

hydrogen bond formation by bulk water and protein may stabilize it at low temperature (Tsai et al., 2002). Therefore, the hydrophilicity of residue 268 may be one of the important reasons for M1-CK maintains activity at low temperature. We mutated 268 residue of M1-CK and RM-CK to glycine, asparagines, aspartic acid, lysine or leucine and examined their specific activities, kinetic properties, thermal inactivation and circular dichroism to find out how residue 268 affect muscle-form CK at low temperature.



Materials and Methods

1. Materials

A. Competent cells:

ECOS™ 101 (Yeastern Biotech, Taipei)

endA1 recA1 relA1 gyrA96 hsdR17(r_k⁻, m_k⁺) phoA supE44 thi-1

$\Delta(lacZYA-argF)U169 \Phi 80 \Delta(lacZ)M15$ F-

ECOS™ 21 (Yeastern Biotech, Taipei)

F- *ompT hsdS β (r β -m β -) dcm gal λ (DE3)*

B. Plasmid:

pET-28a(+) (Novagen, Cat. NO. 69864-3)

C. Chemicals:

NaCl (Merck), NaOH (Merck), MgCl₂ (Merck), HCl (Merck, 37%), Tris

(aMRESCO), KCl (Merck), NiSO₄ (Merck), imidazole (Merck), glucose (Merck),

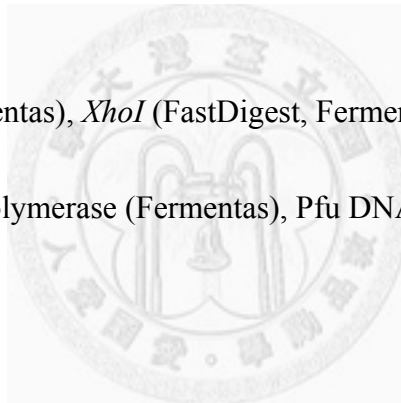
EDTA (Merck), DTT (1,4-dithiothreitol, aMRESCO), kanamycin sulfate

(GIBCO™ Invitrogen), 1-anphthol (Sigma-Aldrich), sodium

p-hydroxymercuribenzoate (Fluka), 2,3-butanedione (diacetyl, Fluka), ADP (potassium salt, Merck), phosphocreatine (PCr, disodium salt, Merck), dNTP (PROTECH), IPTG (aMRESCO), Creatine (anhydrous, Sigma), dithioerythritol (DTE, BDH), ammonium bicarbonate (Sigma), iodoacetamide (IAA, Fluka), acetonitrile (Mallinckrodt CHEMICALS), TFA (SIGMA-ALDRICH), sinapinic acid (SA, Bruker), 25mM ATP solution (EPICENTRE)

D. Enzyme:

NdeI (FastDigest, Fermentas), *XhoI* (FastDigest, Fermentas), T4 DNA ligase (Promega), Taq DNA polymerase (Fermentas), Pfu DNA polymerase (Fermentas), trypsin (SIGMA)



E. Commercial kit:

QIAquick PCR Purification kit (QIAGEN)

Mini-plasmid extraction kit (VIOGENE)

QIAquick Gel Extraction Kit (QIAGEN)

Coomassie plus protein assay reagent (Thermo)

F. DNA agarose gel:

1% : 100ml 1x TBE buffer(aMRESCO)

1g Seakem^R LE Agarose (Lonza)

1.5% : 100ml 1x TBE buffer(aMRESCO)

1.5 g Seakem^R LE Agarose (Lonza)

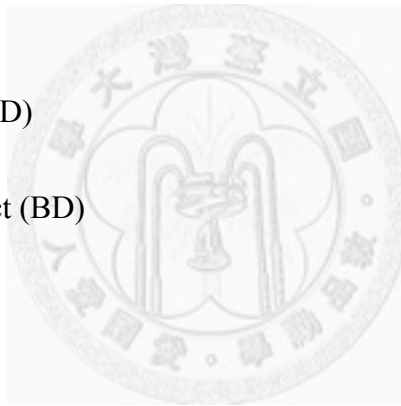
G. LB(1L):

10 g BactoTM trypton (BD)

5 g BactoTM yeast extract (BD)

5 g NaCl

pH7.5



H. SOB , SOC:

SOB(1L) : 20 g BactoTM trypton (BD)

5 g BactoTM yeast extract (BD)

0.5 g NaCl

10 ml 250mM KCl

pH7.0

SOC(100ml) : 100 ml SOB

0.5 ml 2M MgCl₂

2 ml 1M glucose

I. Protein purification buffer:

Charge buffer (8x) : 105.14 g NiSO₄

add distilled water to 1000 ml

Binding buffer (8x) : 2.72 g imidazole

233.76 g NaCl

19.38 g Tris

Add distilled water to 1000 ml

pH 7.9

Wash buffer (8x) : 32.68g imidazole

233.76 g NaCl

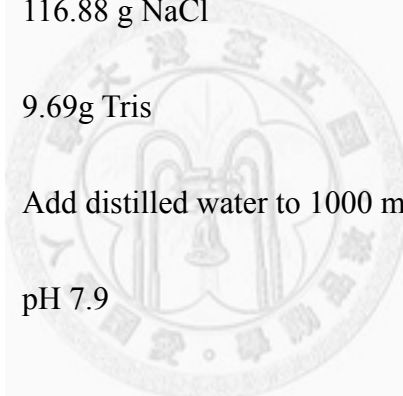
19.38 g Tris

Add distilled water to 1000 ml

pH 7.9

Elute buffer (4x) : 272.32g imidazole
116.88 g NaCl
9.69 g Tris
Add distilled water to 1000 ml
pH 7.9

Strip buffer (4x) : 400g EDTA (Merck)
116.88 g NaCl
9.69g Tris
Add distilled water to 1000 ml
pH 7.9



J. Dialysis buffer:

30 mM Tris

10 mM MgCl₂

1 mM DTT

pH 7.1

K. Protein storage buffer:

Glycerol : 6x buffer = 5:1 v/v

6x buffer: 180 mM Tris

60 mM MgCl₂

6 mM DTT

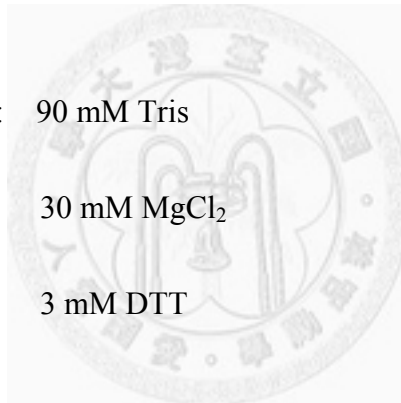
pH 7.1

L. Activity assay reagent:

3x activity assay buffer : 90 mM Tris

30 mM MgCl₂

3 mM DTT



1x activity assay buffer: 1.8 ml glycerol (Merck)

4.2 ml d.d.H₂O

3 ml 3x activity assay buffer

Stop solution (200 ml): 3.607g sodium p-hydroxymercuribenzoate

Add distilled water to 200 ml

5N NaOH 5~10 drops

Colorimetric reaction solution:

0.2g 1-anphthol +10 ml alkaline solution

2,3-butanedione (diacetyl) 1:200, v/v

Alkaline solution(400ml): 24g NaOH

48g Na₂CO₃

Add distilled water to 400 ml

M. 10% SDS-PAGE:

Resolving gel : 25 ml d.d.H₂O

20 ml 30% acrylamide mix (37.5:1, BIO-RAD)

15 ml 4x Tris/SDS (pH8.8)

200 µl 10% AP (Merck)

40 µl TEMED (Merck)

Stacking gel : 12.2 ml d.d.H₂O

2.6 ml 30% acrylamide mix (37.5:1, BIO-RAD)

5 ml 4x Tris/SDS (pH6.8)

100 µl 10% AP (Merck)

20 µl TEMED (Merck)

4x Tris/SDS (pH8.8) : 182g Tris

20 ml 20% SDS (Merck)

Adjust to pH8.8 with 1N HCl

d.d.H₂O add to 1000 ml

4x Tris/SDS (pH6.8) : 12.1g Tris

4ml 20% SDS (Merck)

Adjust to pH6.8 with 1N HCl

d.d.H₂O add to 200 ml

N. SDS-PAGE stain solution (coomassie brilliant blue G-250):

Solution I : 25 % isopropanol (Merck)

10 % acetic acid (Merck)

1.5mg/100ml coomassie brilliant blue G-250 (Merck)

Solution II : 10 % isopropanol (Merck)

10 % acetic acid (Merck)

1.5mg/100ml coomassie brilliant blue G-250 (Merck)

Solution III : 10 % isopropanol (Merck)

3mg/100ml coomassie brilliant blue G-250 (Merck)

2. Methods

A. 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 pET 28a (Wu et al., 2011). M1-CK was constructed previously (Sun et al., 1998), it is also digested with *NdeI* and *XhoI*, and then ligated into pET28a.

To mutate glycine to asparagine, aspartic acid, lysine or leucine at residue 268 of RM-CK, bridge PCR method was used in site directed mutagenesis on RM-CK-pET28a clone. The primers were displayed in Table 2.

Similarly, mutated asparagines to glycine, aspartic acid, lysine or leucine at residue 268 of M1-CK, bridge PCR method was also used in site directed mutagenesis on M1-CK-pET28a clone, the primers were displayed in Table 2.

5 μ l Taq DNA polymerase 10x buffer, 4 μ l dNTP, 32.5 μ l d.d.H₂O, 1 μ l primer (100 μ M) twice, 1 μ l template (RM-CK-pET28a or M1-CK-pET28a), 5 μ l MgCl₂ (25 mM) and 0.5 μ l Taq DNA polymerase are used in the first PCR. The PCR program was one cycle of 94 °C for 5 min, 35 cycles of denature 94 °C for 20 sec, annealing 50 °C for 20 sec and extension 70 °C for 90 sec, and final extension of one cycle at 70 °C for 5 min.

The first PCR products were separated by 1.5 % DNA agarose gel and purified by QIAquick Gel Extraction Kit (QIAGEN). For second PCR, 5 μ l the purified DNA fragment twice, 5 μ l Pfu DNA polymerase 10x buffer, 4 μ l dNTP, 25 μ l d.d.H₂O, 5 μ l MgCl₂ (25 mM) and 1 μ l Pfu DNA polymerase were added. The PCR program was one cycle of 94 °C for 5 min, 15 cycles of denature 94 °C for 20 sec, annealing 52 °C for 20 sec and extension 70 °C for 90 sec, and final extension of one cycle at 70 °C for 5 min.

When second PCR finished, 2 μ l forward and reverse primers were added directly for the third PCR. The PCR program was one cycle of 94 °C for 5 min, 25 cycles of denature 94 °C for 20 sec, annealing 52 °C for 20 sec and extension 70 °C for 90 sec, and final extension of one cycle at 70 °C for 5 min. The third PCR products were purified by QIAquick PCR Purification kit (QIAGEN).

Subsequently, *NdeI* and *XhoI* were used to double digest the third PCR purification products and pET28a vector. 4 μ l d.d.H₂O, 2 μ l 10x Fastdigest buffer, 10 μ l third PCR purification products or pET28a vector, 2 μ l FastDigest *NdeI* and 2 μ l FastDigest *XhoI* were added to the 1.5 ml microcentrifuge tube, then incubated at 37 °C for 20 minutes for digestion. These digested products were subjected to 1 % DNA agarose gel electrophoresis then used QIAquick Gel Extraction Kit (QIAGEN) to purify the sticky-end DNA fragment and pET28a. 3.9 μ l sticky-end DNA fragment, 8 μ l sticky-end pET28a, 1.5 μ l 10x T4 DNA ligase buffer, 1 μ l T4 DNA ligase and 0.6 μ l ATP (25 mM)

are used in ligation at 4 °C overnight.

2 µl ligation product was added to the 100 µl ECOSTM 101 competent cells (storage at -80 °C, Yeastern Biotech, Taipei). Placed on ice for 30 minutes, heat-shocked for 45 seconds at 42 °C water bath then replaced on ice for 2 minutes. 1 ml SOC medium was added to the tubes containing transformed cells, and then incubated at 37 °C for 1 hour with shaking. The transformation cultures were plated on LB/kanamycin plates (LB Agar/Kanamycin 50, RH: 60% ± 15, CMP) and incubated at 37 °C overnight. A single colony was picked and cultured in LB medium with kanamycin overnight (LB : 10 mg/ml kanamycin = 1000:1). Mini-plasmid extraction kit (VIOGENE) was used to purify the recombinant plasmid DNA, then the size of plasmid was confirmed by 1 % DNA agarose gel electrophoresis. After confirming the sequences by commercial company, the recombinant plasmid DNAs were stored at -20 °C.

B. Production of protein of RM-CK and M1-CK mutants

2µl recombinant plasmid DNA was added to 100 µl ECOSTM21 competent cells (storage at -80 °C, Yeastern Biotech, Taipei). Placed on ice for 30 minutes, heat-shocked for 45 seconds at 42 °C water bath then replaced on ice for 2 minutes. 1 ml SOC medium was added to the tubes containing transformed cells, and then incubated at 37 °C for 1 hour with shaking. The transformation cultures were plate on LB/kanamycin

plates (LB Agar/Kanamycin 50, RH: 60% \pm 15, CMP) and incubated at 37 °C overnight.

A single colony was picked and cultured in 100 ml LB medium with 100 μ l kanamycin (10 mg/ml) at 37 °C overnight. Next day, it was added to 900 ml LB with 1000 μ l kanamycin (10 mg/ml), incubated at 37 °C for 4 hour with shaking, and then 1000 μ l IPTG (0.4M) was added, incubated at 37 °C for 1.5 hour with shaking. The culture medium was centrifuged for 30 minutes, supernatant discarded and pellet resuspended in 20 ml binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris, pH 7.9). After sonication (Time: 5 minutes, Pulse: 10, 05, Amp: 1, 30%, SONICS) and centrifuged at 30 minutes at 4 °C, supernatant was filtrated using a 0.2 μ m filter (Sartolab B/T, 150, 0.2 PES, R/FST, Sartorius, made in USA) and applied in to a nickel column.

The nickel column was prepared previous day. The histag binding agarose was purchased from Bioman (Cat No: PBP001.100), and filled with 1x charge buffer overnight. Before protein purification, charge buffer was replaced by 1x binding buffer. The target protein was eluted with elution buffer (0.2 M imidazole, 0.5 M NaCl, 20 mM Tris, pH 7.9), and it was eluted between 200 mM to 300 mM imidazole. The experiment was conducted at 4 °C.

Elution product was dialysed in 30 mM Tris, 10 mM MgCl₂, 1 mM DTT, pH 7.1 at 4

°C for at least 36 hours. The dialysis membrane was purchased from Spectrum Laboratories, Inc. (MWCO:6-8000, reorder no:132 660). After dialysis, the protein was concentrated with an Amicon Ultra 15 filtration tube (MWCO: 10000, Millipore Corp., Billerica, MA, USA). The concentrated protein solution was mixed with protein storage buffer in the ratio of 2/3 (v/v), and storage at -30°C. The final protein storage condition was 30 mM Tris, 10 mM MgCl₂, 1 mM DTT and 50% glycerol. Protein concentration was estimated by Coomassie plus protein assay reagent (Thermo, standard source: bovine serum albumin, concentration: 2.0 mg/ml in a 0.9 % aqueous). The spectrophotometric absorption was measured at 595 nm (Synergy HT ELISA Reader, BIO-TEK).

C. Specific activity assay

M-CK specific activity assay was based on the method described by Hughes and modified to fit different assay conditions (Hughes, 1962). Since the Sigma M-CK activity assay kit (SIGMA Diagnostic, No. 520; Sigma-Aldrich. St Louis, MO, USA), which was used in our previous studies, was no longer available. Therefore, we prepared all chemicals and all stock solution as mention in the Sigma M-CK activity assay kit.

The basal reaction buffer of M-CK activity assay was 30 mM Tris, 10 mM MgCl₂,

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. The pH of the assay buffers were adjusted by titration with HCl, at the assay temperatures.

25 µl 3x activity assay buffer (90 mM Tris, 30 mM MgCl₂, and 3 mM DTT), 25 µl 40mM PCr and 25 µl 3.2 mM ADP were added to 1.5 ml microcentrifuge tube (Axygen, MCT-175-C). After preincubated 15 minutes in water bath, 5 µl enzyme (2.5 µg/ml, dilute with 1x activity assay buffer) was added to the tube, but blank was added 5 µl 1x activity assay buffer. The final reaction condition was 12.5 mM PCr, 1 mM ADP and 0.0125 ng enzyme in the reaction solution of 80 µl. After 15 minutes, the reaction was terminated with 20 µl stop solution (50 Mm p-hydroxyl-mercuribenzoate), then 100 µl α-naphthol (2%, w/v) and 100 µl diacetyl (1:200, v/v) were added for the colorimetric reaction. Finally, 700 µl d.d.H₂O was added and mixed, then incubated at 37°C for 15 minutes. Centrifugation for 5 minutes, 300 µl supernatant added to 96 microwell (Thermo, nuncTM) and spectrophotometric absorption was measured at 520 nm (Synergy HT ELISA Reader, BIO-TEK). Creatine produced in each reaction was quantified using a creatine standard curve (0.08, 0.064, 0.048, 0.032, 0.016, 0, µ mole) and 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.

D. 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, 15 °C or 5 °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 minutes for the 35 °C reaction, 15 minutes for the 25 °C and 15 °C reactions and 45 minutes for the 5°C 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 (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 $v^{-1} = 0$, $(S)^{-1} = -K_m^{-1}$

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.

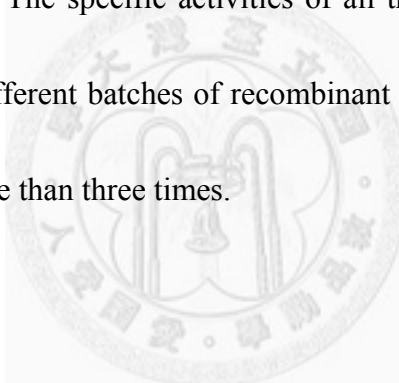
E. Thermal stability assay

Thermal stability experiments were carried out in the activity assay buffer (30 mM Tris, 10 mM MgCl₂, and 1 mM DTT) at pH 7.1 or 8.0. Enzyme was diluted with 1x activity assay buffer to 2.5 µg/ml, and then preincubated at 10, 20, 30, 40, 50, 60 or 70° C for 30 min, then, cooled on ice.

25 µl 3x activity assay buffer (90 mM Tris, 30 mM MgCl₂, and 3 mM DTT), 25µl 40mM PCr and 25µl 3.2 mM ADP were added to 1.5 ml microcentrifuge tube (Axygen, MCT-175-C). After preincubated 15 minutes at 35°C at water bath, 5 µl enzyme was added to the tube, but blank was added 5 µl 1x activity assay buffer. The final reaction condition was 12.5 mM PCr, 1 mM ADP and 0.0125 ng enzyme in the reaction solution of 80 µl. After 15 minutes reaction at 35° C, the reaction was terminated with 20 µl stop solution (50 Mm p-hydroxyl-mercuribenzoate), then 100 µl α-naphthol (2%, w/v) and

100 μl diacetyl (1:200, v/v) were added for the colorimetric reaction. Finally, 700 μl d.d.H₂O was added and well mixed, then incubated at 37°C for 15 minutes. Centrifugation for 5 minutes, 300 μl supernatant added to 96 microwell (Thermo, nuncTM) and spectrophotometric absorption was measured at 520 nm (Synergy HT ELISA Reader, BIO-TEK). Creatine produced in each reaction was quantified using a creatine standard curve (0.08, 0.064, 0.048, 0.032, 0.016, 0, μ mole)

At least three 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.



F. Circular dichroism

Circular dichroism (CD) spectra were recorded on a Jasco J715 spectropolarimeter (Jasco International, Tokyo, Japan) 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 (Hellma Analytics, order number: 165-1-40, type: 165-QS) was used at a scan speed of 20 nm \cdot min⁻¹ from 200 nm to 250 nm. The solution for CD contained 2.32 μM of M-CK protein in 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.

G. Structure modeling

Since the crystal structure of RM-CK and RM-CK G268N had been resolved (Rao et al., 1998; Wu et al., 2011), and the primary sequences of RM-CK and M1-CK share 86% identity, therefore, we could use RM-CK as template to modeling the 3D structure of RM-CK mutants, M1-CK and M1-CK mutants.

Since M-CK is highly conserved enzyme, the homology modeling methods was used to model the structure of our target proteins (Marti-Renom et al., 2000), and the MODELLER is the free program to carry out homology modeling for model building (Eswar et al., 2007; <http://salilab.org/modeller/>). The template for build structure was the PDB 2CRK (Rao et al., 1998). The output 3D structures were displayed by the free program included PyMol (DeLano, W.L. The PyMOL Molecular Graphics System (2002) DeLano Scientific, San Carlos, CA, USA; <http://www.pymol.org/>), Swiss-PdbViewer (Guex and Peitsch, 1997; <http://www.expasy.org/spdbv/>). The solvent accessible surface of each residue was calculated by free program MolMol (Koradi et al., 1996), and the $C\alpha$ distances were calculated by Swiss-PdbViewer.

Results

A. Construction of site-directed mutagenesis of RM-CK and M1-CK mutants

Using RM-CK-pET28a or M1-CK-pET28a as template, the 268 residue mutants were constructed by overlap-extension technique. The PCR product and pET28a were double digestion with NdeI and XhoI. After purified by gel extraction, the DNA fragment was ligated to the pET28a vector (Fig. 1). These resulting plasmids were designated as RM-CK G268N, RM-CK G268D, RM-CK G268K, RM-CK G268L, M1-CK N268G, M1-CK N268D, M1-CK N268K, M1-CK N268L. The inserted DNA fragment was 1146 base pairs; pET28a was 5369 base pairs and the total size of recombinant plasmid was 6834 base pairs(Fig 1). The plasmids were storage at -20 °C.

B. Production of protein of RM-CK and M1-CK mutants

The recombinant plasmids were transformed in to ECOSTM21 competent cells to express the recombinant protein. The protein were applied in to a nickel column and eluted with elution buffer (0.2 M imidazole, 0.5 M NaCl, 20 mM Tris, pH 7.9). The recombinant protein appeared at 200 mM and 300 mM imidazole (Fig. 2). All the mutant proteins are the same size as the wild-type protein, about 45 kDa (Fig. 3). After

dialysis in 30 mM Tris, 10 mM MgCl₂, 1 mM DTT, pH 7.1 at 4 °C, the proteins were concentrated with Amicon Ultra 15 filtration tubes. The concentrated protein solution was mixed with protein storage buffer and storage at -30 °C. The final protein storage condition was 30 mM Tris, 10 mM MgCl₂, 1 mM DTT and 50% glycerol.

C. Specific activity assay

Polar side chain of residue 268 of muscle form creatine kinase show higher activity at low temperature

The residue 268 of M1-CK and RM-CK were mutated to glycine, asparagine, aspartic acid, lysine or leucine, individually. M-CK specific activity assay was based on the method described by Hughes and modified to fit different assay conditions (Hughes, 1962). The specific activities were assayed at pHs 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.

The four M1-CK mutants showed different pH-temperature specific activity patterns compared to wild-type M1-CK (Fig 4). M1-CK, M1-CK N268D, M1-CK N268G, M1-CK N268K and M1-CK N268L showed their highest specific activity of 445 ± 42.44 U, 446 ± 40.75 U, 385 ± 41.48 U, 364 ± 22.27 and U 305± 32.2 (mean ± S.D.), respectively, at pH 7.1 and 30 °C (Table 4).

At pH 7.1 and 7.4, M1-CK had highest activity at 30 °C, and started to decrease at

temperature > 30 and <30 °C, but still remain >100 U activity at 15 and 10 °C. At pH 7.7, M1-CK had highest activity of 151 ± 4.82 at 30 °C and 143 ± 7.4 U at 15 °C, and still remain 95.5 ± 5.54 U at 10 °C. At pH 8.0, M1-CK was able to maintain its activity around 70 U at 15 and 10 °C. Activity trend of M1-CK N268G was similar to M1-CK at pHs 7.1, 7.4 and 7.7, but lower at all temperature. At pH 8.0, compared to M1-CK, M1-CK N268G was 22% and 34% less active at 15 and 10 °C, respectively. M1-CK N268D was the mutant enzyme of highest activity at 35 and 30°C at all pHs, and maintained activity of 127 ± 3.2 and 90.6 ± 3.87 at 15 and 10 °C, respectively at pH 7.7. At pH 8.0, compared to M1-CK, M1-CK N268D was 11.5 % and 13 % less active at 15 and 10 °C, respectively. The activity of M1-CK N268K was lower than M1-CK N268D and almost the same as M1-CK N268G at 15 °C at either pH 7.7 or 8.0 and at pH 8.0, at 10 °C. At pH 8.0, at 10 °C, activity of M1-CK N268K was lower than M1-CK N268D and higher than M1-CK N268G, and it was 24% less activity compared to M1-CK. M1-CK N268K and M1-CK N268D could recover activities at 15 °C of all pHs and at 10 °C, pH 8.0. M1-CK N268L showed the same activity pattern as M1-CK N268G at low temperature. M1-CK N268L showed the lowest activity and was 62.8 %, 39.7 %, 55 % and 67 % less activity compared to M1-CK at 15 and 10 °C at either pH 7.7 or 8.0, respectively.

The four RM-CK mutants also showed different pH-temperature specific activity

patterns compared to the wild-type (Fig 5). RM-CK, RM-CK G268N, RM-CK G268K, RM-CK G268D, and RM-CK G268L showed their highest specific activity of 352.76 ± 24 U, 436.5 ± 7.1 U, 407.6 ± 1.1 U, 401.2 ± 3.7 U, and 314.5 ± 4.6 U (mean \pm S.D.), respectively, at pH 7.1 at 35 °C (Table 5).

RM-CK had higher activity around 35 to 25°C and decreases as temperature decreased. At pH 8.0 at 10°C, RM-CK lost 92% of its activity (29 ± 3.1 U). RM-CK G268N showed the similar activity pattern as RM-CK at high temperature (40 to 25 °C), but had higher activity than RM-CK at low temperature (15 and 10°C) at pHs 7.7 and 8.0. At pH 7.7, RM-CK G268N showed 50% higher activity compared to RM-CK at 10°C. RM-CK G268N, as M1-CK, maintained its activity of 66.2 ± 7.6 U and showed 56% higher activity compared to RM-CK at pH 8.0, at 10°C. RM-CK G268D and RM-CK G268K had higher activities than RM-CK and RM-CK G268L at all pHs and temperatures, and their activities were similar to RM-CK G268N excepted at 10°C, at pHs 7.7 and 8.0. The activity of RM-CK G268L was the same as RM-CK at pH 7.7, at low temperature (15 to 5°C). At pH 8.0 at 10°C, RM-CK G268L showed lowest activity than other.

A polar side chain of residue 268 of muscle-form creatine kinase showed higher activity than non-polar residue at low temperature (15 and 10 °C), at high pHs (7.7 and 8.0).

D. Kinetic assay

Polar side chain of residue 268 could maintain stable K_m and K_{cat} or increased K_{cat} .

The kinetic parameters, K_m^{ADP} , K_m^{PCr} , K_{cat}^{ADP} , K_{cat}^{PCr} , of each enzyme were assayed at 35, 25, 15 and 5°C at either pH 7.1 or 8.0.

The K_m^{ADP} trend of M1-CK N268G resembled that of M1-CK N268K at pH 7.1, but lowers all temperature (Fig 6). At pH 7.1, M1-CK, M1-CK N268D and M1-CK N268L had similar K_m^{ADP} pattern. At pH 8.0, M1-CK N268G, M1-CK N268K and M1-CK N268L showed similar K_m^{ADP} pattern and M1-CK N268L was the lowest. M1-CK kept its K_m^{ADP} at different temperatures but rose slightly at 5°C at pH 8.0. M1-CK N268D had similar K_m^{ADP} to others except at 15°C, at pH 8.0.

At pH 7.1, the K_m^{ADP} of RM-CK and its mutants were not significantly different at all temperatures except RM-CK G268D was lower than RM-CK and other mutants at 35°C (Fig 8). At pH 8.0, RM-CK G268K resembled that of RM-CK G268L and higher at all temperatures, both of these two mutants maintained their K_m^{ADP} at 35, 25 and 15°C but decreased at 5°C. The K_m^{ADP} of RM-CK and RM-CK G268D decreased at 25°C then maintained it as temperature decreased. RM-CK G268N maintained its K_m^{ADP} at pH 8.0 at all temperatures.

At pH 7.1, the K_m^{PCr} of M1-CK and its mutants increased as temperature decreased (Fig 6). At pH 7.1, from 25 to 5°C, M1-CK N268G had highest K_m^{PCr} and M1-CK had

lowest K_m^{PCr} . M1-CK N268D, M1-CK N268K and M1-CK N268L were not significantly different at pH 7.1 from 25 to 5°C. At pH 8.0, M1-CK N268G showed no significant difference with M1-CK N268D at all temperatures, and M1-CK N268L was higher than them at 25 and 15°C. At pH 8.0, M1-CK was slightly decreased as temperature decreased, but generally speaking, maintained its K_m^{PCr} at all temperatures. M1-CK N268K showed highest K_m^{PCr} at 35 and 25°C but decreased at 15 °C at pH 8.0.

At pH 7.1, the K_m^{PCr} of RM-CK and its mutants increased as temperature decreased except RM-CK G268N (Fig 8). At pH 7.1, RM-CK G268N maintained its K_m^{PCr} at 15 and 5°C. At pH 8.0, the K_m^{PCr} of RM-CK and RM-CK G268N decreased slightly as temperature decreased, on the other hand, those of RM-CK G268K and RM-CK G268L also decreased slightly as temperature decreased but increased at 5°C. At pH 8.0, K_m^{PCr} of RM-CK G268D decreased more significantly than other mutants when temperature decreased. K_m^{PCr} of RM-CK and mutants were no significantly different at 15°C, pH 8.0.

At pH 7.1, the K_{cat}^{ADP} of M1-CK and mutants decreased as temperature decreased, but, unlike other four mutants decreased sharply, M1-CK decreased gently (Fig 7). At pH 8.0, M1-CK could maintain its K_{cat}^{ADP} when temperature changed; K_{cat}^{ADP} of M1-CK N268G decreased slightly as temperature decreased. The K_{cat}^{ADP} of M1-CK N268D, M1-CK N268K and M1-CK N268L undulated at pH 8.0.

At pH 7.1, K_{cat}^{ADP} of RM-CK and mutants decreased as temperature decreased, and there was no significant difference at 25, 15 and 5°C (Fig 9). At pH 8.0, K_{cat}^{ADP} of RM-CK and mutants also decreased as temperature decreased, and there was not significant difference at 35 and 5°C. At pH 8.0, at 25 and 15 °C, they also showed the same K_{cat}^{ADP} except that of RM-CK G268K was higher than other.

The K_{cat}^{PCr} of M1-CK and mutants decreased as temperature decreased, at pH 7.1, and compared to other mutants, M1-CK decreased more gently (Fig 7). The K_{cat}^{PCr} of M1-CK N268D resembled that of M1-CK N268K at pH 7.1. At pH 8.0, M1-CK could maintain its K_{cat}^{PCr} as temperature decreased; M1-CK N268G also could maintain its K_{cat}^{PCr} at 35, 25 and 15°C, but decreased at 5°C. At pH 8.0, K_{cat}^{PCr} of M1-CK N268D, M1-CK N268K and M1-CK N268L were decreased as temperature decreased, but at 25°C, K_{cat}^{PCr} of M1-CK N268K and M1-CK N268L increased significantly.

The K_{cat}^{PCr} of RM-CK and mutants decreased as temperature decreased, at pH 7.1, and RM-CK G268K was not significantly different with RM-CK G268L at all temperature (Fig 9). At pH 8.0, K_{cat}^{PCr} of RM-CK and mutants also decreased as temperature decreased.

At pHs 7.1 and 8.0, M1-CK and RM-CK G268N could maintain relatively stable K_m and K_{cat} at all temperature. The K_m and K_{cat} of M1-CK N268D, RM-CK G268D, M1-CK N268K and RM-CK G268K varied at pH 8.0, but, M1-CK N268K and RM-CK

G268K, showed higher values of K_{cat} at pH 7.1 and 8.0. RM-CK and M1-CK N268G also had relative low variation of K_m and K_{cat} , but compared to M1-CK and RM-CK G268N, their variations were more significantly. The K_m and K_{cat} of M1-CK N268L and RM-CK G268L were more similar to M1-CK N268K and RM-CK G268K at most conditions, but always lower. Generally speaking, polar side chain of residue 268 could maintain stable K_m and K_{cat} as temperature change or with increased K_{cat} .

E. Thermal stability assay

Polar side chain of residue 268 of M-CK were more stable than non-polar mutants

Thermal stability experiments were carried out at pHs 7.1 or 8.0. Enzyme was incubated at 10, 20, 30, 40, 50, 60 or 70 °C for 30 minutes before assay at 35°C. The highest specific activity was taken as 100%, and when the activity decreased to 50%, the temperature was called denatured temperature (Zhao et al., 2006a).

At pH 7.1, RM-CK G268N and all other mutants exhibited denatured temperatures between 37 to 40 °C, while that of the wild type was 42 °C (Fig 10). At pH 8.0, the thermal stability of polar side chain of residue 268 improved. RM-CK G268N, RM-CK G268D and RM-CK G268K exhibited denatured temperatures between 32 to 37 °C at pH 8.0, whereas those of RM-CK and RM-CK G268L were between 23 to 28 °C at pH 8.0.

M1-CK and its mutants, except M1-CK N268L, exhibited denatured temperatures between 40 to 45°C at pH 7.1 and between 32 to 37 °C at pH 8.0 (Fig 11). M1-CK N268L exhibited denatured temperatures between 32 to 37 °C at pH 7.1 and between 25 to 30°C at pH 8.0.

F. Circular dichroism

Secondary structures were no significant change between wild type and mutant proteins

Circular dichroism (CD) spectra were recorded at pH 7.1 or 8.0 at temperatures 35 °C, 25 °C, 15 °C or 5 °C (Kelly et al., 2005).

M1-CK and its mutants could maintain their secondary structures at 7.1 or 8.0 at all temperature (Fig 12). At pH 7.1, M1-CK and M1-CK N268L showed different CD pattern than other mutants at 25 and 5 °C. At pH 8.0 at 25 °C, M1-CK showed different CD pattern than other mutants. RM-CK and its mutants could maintain their secondary structures during temperature change expect RM-CK G268K. RM-CK G268K could not maintain its secondary structures as temperature decreased (Fig 13).

G. Structure modeling

Continued hydrophilic region was formed by the connection of hydrophilic residue

268

Analysis of the structure of RM-CK, M1-CK and their mutants, the residue 268 was located at the outside of the enzyme, and the side chain was point out to the solvent.

The PyMol program showed the hydrophilic and hydrophobic surface around residue 268 of RM-CK and it mutants (Fig 14). When the residue 268 was hydrophilic, as in RM-CK G268N, RM-CK G268D and RM-CK G268K, their hydrophilic surfaces could connect to two hydrophilic residue, Lys¹³⁸ and His²⁶⁹, forming a continuous hydrophilic region including two clusters of hydrophilic residues, Lys¹³⁸ to Arg¹⁵² and Gln²⁵⁸ to Lys²⁶⁶, and this two hydrophilic cluster could also connect to other hydrophilic region. By the connection of hydrophilic residue 268, the surface of enzyme could form continuous hydrophilic region, which in turn is easier to form hydrogen bond with water. On the other hand, when the residue 268 was hydrophobic, as in RM-CK and RM-CK G268L, there would be a hydrophobic spot on the surface, and this hydrophobic spot could disrupt the connection with other hydrophilic region.

We also analyzed the hydrophilic and hydrophobic surface around residue 268 of M1-CK and it mutants (Fig. 15). When the residue 268 is hydrophilic, as in M1-CK, M1-CK N268D and M1-CK N268K, it could form a hydrophilic spot that connected to

the hydrophilic residue Lys¹³⁸, thus it could connect to the clusters of hydrophilic residues between Asp⁷⁸ to Glu⁸³. By connecting to residues His²⁶⁹ and His²⁶⁷, residue 268 could also strengthen hydrophilic connection between Tyr¹⁴⁰ to Arg¹⁵² and Gln²⁵⁸ to His²⁶⁹. When the residue 268 was hydrophobic, as in M1-CK N268G and M1-CK N268L, it formed a hydrophobic spot, and could not form continuous hydrophilic region.

The hydrophilic residues around residue 268 were exposed more to solvent or moved closely to residue 268

The solvent accessible surface (SAS) of each residue was calculated by MolMol. The SAS of hydrophilic residues around residue 268 were adjusted by the side chain of residue 268 at RM-CK, M1-CK and their mutants (table 10). RM-CK G268N, RM-CK G268D and RM-CK G268K had higher SAS at Lys¹³⁸ than RM-CK and RM-CK G268L. The clusters of hydrophilic residues, Lys¹³⁸ to Arg¹⁵², was also had higher SAS in RM-CK G268N, RM-CK G268D and RM-CK G268K. The SAS of His²⁶⁹ and the hydrophilic cluster, Gln²⁵⁸ to His²⁶⁹, were also increased in RM-CK mutants with polar residue 268, except RM-CK G268K. In M1-CK and its mutants, the SAS of hydrophilic cluster, Asp⁷⁸ to Glu⁸³, were higher when the residue 268 was polar, as in M1-CK, M1-CK G268D and M1-CK G268K (table 10). Generally speaking, the hydrophilic

region that connected by polar residue 268 always exposed more area to the solvent.

Using Swiss-PdbViewer to analysis the C α distances of residue 268 to nearby hydrophilic residues. Three clusters of hydrophilic residues between Asp⁷⁸ to Thr⁸³, Glu¹⁶⁰ to Tyr¹⁷⁴ and His²⁵⁹ to His²⁶⁹ were shifted closer to the polar residue 268 in RM-CK G268N, RM-CK G268D and RM-CK G268K (table 11). In M1-CK and its mutants, when the residue 268 was hydrophilic, as in M1-CK, M1-CK N268D and M1-CK N268K, two clusters of hydrophilic residues between Glu¹⁶⁰ to Lys¹⁷² and Gln²⁵⁸ to His²⁶⁹ were shifted closer to residue 268 (table 13). The hydrophilic clusters nearby residue 268 came together when the side chain of residue 268 was hydrophilic.

Compared to M1-CK N268G and RM-CK, active site residues of other mutants

shifted closer to the active site center

The crystal structure of RM-CK G268N shows a small active site pocket (Wu et al., 2011). We analyzed the active site pocket of all mutants by C α distances to the active site center (residue 283).

When the residue 268 of RM-CK and M1-CK was polar, the flexible loop, residues 63 to 74, shifted closer to residue 283 (table 14, table 15). The active site residues of M1-CK N268G and RM-CK were always further from residue 283. Compared to the wild type, arg¹³⁰, arg¹³², glu²³², arg²³⁶, arg³²⁰ of RM-CK mutants moved closer to residue

283 (table 14). The small α helix of residues 284 to 286 was closer to the active site center when the residue 268 of RM-CK was polar. For M1-CK and its mutants, the $C\alpha$ distances of arg¹³⁰, arg¹³², arg²³⁶ to residue 283 were shorter than in M1-CK N268G (table 15). The flexible loop, residues 230 to 236, shifted closer to the active site center in M1-CK, M1-CK N268D and M1-CK N268K.



Discussion and Conclusion

Comparing the activity of M1-CK and RM-CK mutants at different temperature and pH, the mutants with polar side chain of residue 268 had higher activity than mutants with non-polar side chain of residue 268 under most conditions. M1-CK and RM-CK G268N could maintain their activity under all conditions, especially at low temperature and higher pH. M1-CK N268D, M1-CK N268K, RM-CK G268D and RM-CK G268K had similar activity pattern and they also could maintain their activity at low temperature as M1-CK and RM-CK G268N, but always lower. Analyzing the crystal structures of RM-CK and RM-CK G268N, the side chain of residue 268 locates at the surface of enzyme and points out to the solvent. Therefore, interaction between the polar side chain of residue 268 and water may play an important role in conferring the appropriate 3D structures for CK to function well at low temperature.

From the kinetic assay results, M1-CK and RM-CK G268N showed least variations, both of them could maintain stable K_m and K_{cat} values at all assay temperature and pH. Compared to M1-CK and RM-CK G268N, mutants with charge side chain aspartic acid or lysine, became undulated in kinetic parameters at pH 8.0. Compared to wild type, RM-CK G268D showed higher ADP affinity and lower PCr affinity, and M1-CK N268D showed similar tendency. Examine the modeling structure of RM-CK G268D,

the flexible loop, residues 63 to 74, moved closer to the active site center. This flexible loop controls the entrance of PCr, therefore, PCr may become more difficult to enter the active site in RM-CK G268D. While residues 130, 132 and 320, which bind ADP, moved closer to the active site, and may increase ADP binding affinity. In RM-CK G268K, the flexible loop that controls the entrance of substrate and the active site residues moved closer to the active center residue 283, while these residues moved away from residue 283 in M1-CK N268K. As shown in the kinetic assay results, M1-CK N268K and RM-CK G268K showed lower or similar ADP and PCr affinity to the wild type.

K_{cat} values were also highly influenced by charged side chain at residue 268. The aspartic acid side chain of RM-CK G268D should be negative by charged at all assay pH, showed lower K_{cat} than the wild type. While the lysine side chain of RM-CK G268K should be positive by charged at all assay pH, showed higher K_{cat} than the wild type. RM-CK G268N and RM-CK G268L showed similar K_{cat} to the wild type. M1-CK and its mutants also showed similar tendency of K_{cat} . We examined the electrostatic surface of the modeling structure, when residue 268 was aspartic acid, the surface became more negative. On the other hand, when residue 268 was lysine, the negative electrostatic around residue 268 decreased. Surface electrostatic may influence the local surrounding pH, and asp²⁶⁸ may increase the local surrounding pH while lys²⁶⁸ decrease

the local surrounding pH. At higher pH, the reaction of RM-CK may become more random (Morrison and James, 1965). These may be the reasons for aspartic acid mutants showed lower K_{cat} and lysine mutants showed higher K_{cat} .

From the circular dichroism (CD) spectra, the secondary structures of all mutants were not significantly changed as predicted previously (Wu et al., 2011). The thermal stability data showed that RM-CK G268N, RM-CK G268D and RM-CK G268K were more stable than RM-CK and RM-CK G268L at pH 8.0. M1-CK N268L was most unstable than wild type and other mutants at pH 7.1 and pH 8.0. This result suggests that polar mutants were more stable than non-polar mutants, this mean, the hydrogen bond and intra-molecular hydrophobic interaction were stronger in polar mutants.

Cold-induced change in a protein based on the very unusual feature of hydrophobic interaction. At low temperature, protein may be in a cold denatured state, and hydrophobic interaction decreases as a result of the increase in hydration contribution (Privalov, 1990). Polar rather than non-polar surfaces promote formation of hydrogen bonding network at low temperature (Robinson and Cho, 1999). The hydrogen bond formation by bulk water and protein may stabilize the protein at low temperature (Tsai et al., 2002). Therefore, without binding water at low temperature, enzyme may be unstable and lost its function. The stable structure contribution by hydrogen bond between polar residue and water may be the reason for mutants with polar side chain of

residue 268 had higher activity at low temperature.

Since the side chain of residue 268 of RM-CK located on the surface of the molecule and in contact with the solvent, the hydrophilic property may be the reason for the enzyme with polar residue 268 to still exhibit activity at low temperature and structurally more stable than non-polar mutants. Hydrophilic side chains located at molecular surface, where a polar atom can easily form a hydrogen bond with the solvent may reduce the hydrophobicity on the interface of protein and water (Fersht, 1985; Timasheff, 1993; Xu et al., 1997).

We examined the hydrophilic and hydrophobic surface of RM-CK, M1-CK and their mutants by molecular modeling. When the side chain of residue 268 was polar, it formed continuous hydrophilic region with nearby hydrophilic residues, and this hydrophilic region is easy to form hydrogen bonds with bulk water, which could stabilize the enzyme. Then, we were using the program to analysis the SAS and C_{α} distances of hydrophilic residues that nearby residue 268. From this analysis, we could find that these hydrophilic residues exposed more area to solvent or moved closer to residue 268. This phenomenon may cause by the connected hydrogen bonds. When the hydrophilic region that includes residue268 interacts with surrounding water to form a network hydrogen bonds, these interactions may move hydrophilic residues closer.

At low temperature, water prefers to interact with water; this condition decreased

inter-molecular hydrogen bond between water and hydrophilic residue of enzyme surface and also decreased intra-molecular hydrophobic interaction. Therefore, enzyme was unstable at low temperature. When residue 268 was polar, it formed continuous hydrophilic region with nearby hydrophilic residues, and increased the attraction of hydrophilic residues to water. The hydrogen bond formation by water and protein surface increased inter-molecular hydrophilic interaction and increased intra-molecular hydrophobic interaction. Thus, by interaction with polar residue and water, the enzyme became more stable and result in maintained activity at low temperature.

By simple site directed mutagenesis, we provide a new insight to the enzyme structures and function at low temperature. The hydrogen bond formation may be the critical reason for enzyme maintained its function at low temperature. This study may also provide a clue to understand the possible evolution of cold adapted enzyme.

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		20		40	
RM-CK	MPFGNTHNKY	KLNYKSEEEY	PDLSKHNNHM	AKVLTDPDYK	KLRDKETPSG 50
M1-CKNFSVDD.F	...A.....KEM.GQ.ST. 50
	60		80		100
RM-CK	FTLDDVIQTG	VDNPGHPFIM	TVGCVAGDEE	SYTVFKDLFD	PIIQDRHGGF 100
M1-CKA.....E.....	.V.S.....Y 100
	120		140		
RM-CK	KPTDKHKTDL	NHENLKGDD	LDPHYVLSSR	VRTGRS IKGY	TLPPhCSRGE 150
M1-CK	.A.....	.F.....	...N.....	A...N.... 150
	160		180		200
RM-CK	RRAVEKLSVE	ALNSLTGEFK	GKYYPLKSM	EQEQQLIDD	HFLFDKPVSP 200
M1-CKD.....	DA..E..A. 200
	220		240		
RM-CK	LLLASGMARD	WPDARGIWHN	DNKSFLVWVN	EEDHLRVISM	EKGGNMKEVF 250
M1-CKA.....	E..T.....	QP..... 250
	260		280		300
RM-CK	RRFCVGLQKI	EEIFKKAGHP	FMWNEHLGYV	LTCPSNLGTG	LRGGVHVKLA 300
M1-CKR.HN.GFIP 300
	320		340		
RM-CK	HLSKHPKFEE	ILTRLRLQKR	GTGGVDAAV	GSVFDISNAD	RLGSSEVEQV 350
M1-CK	K..T.A..D.S.	.G.....	.I.....D.. 350
	360		380		
RM-CK	QLVVDGVKLM	VEMEKKLEKG	QSIDDMIPAQ	K* 382	
M1-CK	.C.....	I.....	E...S.....	.. 382	

Table 1. The primary structures of RM-CK and carp M1-CK share 86% identity. Dot identified the same as above.

SOURCE ORGANISM	NAME	THE POSITIONS OF THE AMINO ACID				ACCESSION
		112	267	268	270	
<i>Oryctolagus cuniculus</i>	Creatine kinase muscle	H	A	G	P	NP_001075708.1
<i>Homo sapiens</i>	muscle creatine kinase	–	–	–	–	NP_001815.2
<i>Pan troglodytes</i>	muscle creatine kinase isoform 1, 2	–	–	–	–	XP_001163628
<i>Rattus norvegicus</i>	skeletal muscle creatine kinase	–	–	–	–	AAA40935.1
<i>Mus musculus</i>	Creatine kinase muscle	–	–	–	–	AAI32425.1
<i>Sus scrofa</i>	muscle creatine kinase	–	–	–	–	NP_001123421.1
<i>Bos taurus</i>	muscle creatine kinase	–	–	–	–	AAX31355.1
<i>Equus caballus</i>	similar to muscle creatine kinase	–	–	–	–	XP_001502572.1
<i>Canis lupus</i>	Creatine kinase M-type	–	–	–	–	P05123.3
<i>Torpedo californica</i>	Creatine kinase M-type	Q	–	–	G	P04414.1
<i>Physeter catodon</i>	cytoplasmic creatine kinases	–	–	–	–	BAF63632.1
<i>Gallus gallus</i>	M-CK	–	–	–	–	NP_990838.1
<i>Xenopus laevis</i>	muscle creatine kinase	F	–	–	–	NP_001080073.1
<i>Zaocys dhumnades</i>	creatine kinase	–	–	–	–	AAF76899.1
<i>Danio rerio</i>	muscle creatine kinase a	F	H	N	G	NP_571007.1
<i>Danio rerio</i>	muscle creatine kinase b	W	H	N	G	NP_001099153.1
<i>Cyprinus carpio</i>	creatine kinase M1-CK	F	H	N	G	AAC96092.1
<i>Cyprinus carpio</i>	creatine kinase M2-CK	F	H	N	G	AAC96093.1
<i>Cyprinus carpio</i>	creatine kinase M3-CK	W	H	N	G	AAC96094.1
<i>Salmo salar</i>	creatine kinase-1, 2, 3, 4	F	H	N	G	ACH70913.1
<i>Ictalurus punctatus</i>	creatine kinase	W	H	N	G	AAO25756.1
<i>Ictalurus punctatus</i>	creatine kinase	F	H	N	G	AAO25755.1
<i>Oreochromis mossambicus</i>	muscle-type creatine kinase CKM1	–	H	D	G	AAK56447.1
<i>Oreochromis mossambicus</i>	muscle-type creatine kinase CKM2	F	H	N	G	AAK56448.1
<i>Chaenocephalus aceratus</i>	creatine kinase muscle isoform 1	F	H	N	G	AAO24738.1
<i>Chaenocephalus aceratus</i>	creatine kinase muscle isoform 2	F	H	–	G	AAO24739.1
<i>Lethenteron japonicum</i>	creatine kinase 1	P	K	–	–	BAD11138.1
<i>Lethenteron japonicum</i>	creatine kinase 2	P	K	–	–	BAD11139.1
<i>Myxine glutinosa</i>	muscle creatine kinase	P	K	–	–	AAP68792.1
<i>Eptatretus stoutii</i>	muscle creatine kinase	P	K	–	–	AAP68793.1
<i>Ciona intestinalis</i>	creatine kinase	P	S	–	E	NP_001027661.1

Table 2. Amino acid sequences of muscle-form creatine kinase of different species. The table showed residue 112, 267, 268, 270. Dash identified the same residue as *Oryctolagus cuniculus*.

	Forward and reverse primers	internal primers for mutation sites
RM-CK	5'-cgcgccggcatatgccgttc-3'	5'-cacatgaaggggtggttagctttc-3'
G268N	5'-ggccgctcgagctacttctg-3'	5'-ctttaagaaagctaaccacccc-3'
RM-CK	5'-cgcgccggcatatgccgttc-3'	5'-ttaagaaagctgaccacccttcatg-3'
G268D	5'-ggccgctcgagctacttctg-3'	5'-catgaaggggtggtcagctttcttaa-3'
RM-CK	5'-cgcgccggcatatgccgttc-3'	5'-ttaagaaagctaagcacccttcatg-3'
G268K	5'-ggccgctcgagctacttctg-3'	5'-catgaaggggtgcttagctttcttaa-3'
RM-CK	5'-cgcgccggcatatgccgttc-3'	5'-ttaagaaagctctccacccttcatg-3'
G268L	5'-ggccgctcgagctacttctg-3'	5'-catgaaggggtggagagctttcttaa-3'

	Forward and reverse primers	internal primers for mutation sites
M1-CK	5'-gggcccataatgcctttcggaacaccac-3'	5'-cacatgaaggggtggttagctttc-3'
N268G	5'-gggcctcgagtacttctgggcagggatc-3'	5'-ctttaagaaagctaaccacccc-3'
M1-CK	5'-gggcccataatgcctttcggaacaccac-3'	5'-ttaagaaagctgaccacccttcatg-3'
N268D	5'-gggcctcgagtacttctgggcagggatc-3'	5'-catgaaggggtggtcagctttcttaa-3'
M1-CK	5'-gggcccataatgcctttcggaacaccac-3'	5'-ttaagaaagctaagcacccttcatg-3'
N268K	5'-gggcctcgagtacttctgggcagggatc-3'	5'-catgaaggggtgcttagctttcttaa-3'
M1-CK	5'-gggcccataatgcctttcggaacaccac-3'	5'-ttaagaaagctctccacccttcatg-3'
N268L	5'-gggcctcgagtacttctgggcagggatc-3'	5'-catgaaggggtggagagctttcttaa-3'

Table 3. The primer sequences used to constructe site-directed mutagenesis on residue 268 of RM-CK and M1-CK mutants.

specific activity (U)				
pH 7.1				
Temperature (°C)	40	35	30	25
M1-CK	210.27 ± 3.9	213.17 ± 3.93	444.9 ± 42.4	306.4 ± 44.99
M1-CK N268G	170.54 ± 25	205 ± 2.73	384.6 ± 41.5	298.3 ± 14.45
M1CK N268D	160.63 ± 16	384.72 ± 26.8	446.44 ± 40.8	226.8 ± 18.43
M1-CK N268K	155.88 ± 14	355.19 ± 25.1	364.02 ± 22.3	208.1 ± 20.06
M1-CK N268L	253.02 ± 35	239.95 ± 20.4	305.05 ± 32.2	340.9 ± 6.735
Temperature (°C)	20	15	10	5
M1-CK	222.9 ± 15.4	207.7 ± 31.05	109.32 ± 3.5	40.47 ± 4.27
M1-CK N268G	215.3 ± 7.42	185.3 ± 7.587	99.863 ± 7.62	38.5 ± 2.82
M1CK N268D	214 ± 16.7	168.3 ± 10.93	34.705 ± 1.96	37.25 ± 2.57
M1-CK N268K	208.8 ± 13.4	176.3 ± 10.84	44.192 ± 5.3	35.84 ± 3.15
M1-CK N268L	164.1 ± 17.8	130.2 ± 16.2	98.687 ± 1.34	51.72 ± 2.09

specific activity (U)				
pH 7.4				
Temperature (°C)	40	35	30	25
M1-CK	149.59 ± 11	199.49 ± 13.6	263.96 ± 13.4	238.3 ± 20.86
M1-CK N268G	119.42 ± 22	170.37 ± 3.22	231.17 ± 19.1	220.5 ± 18.41
M1CK N268D	93.033 ± 6.7	295.16 ± 6.4	304.4 ± 13.2	168 ± 13.26
M1-CK N268K	85.88 ± 7.8	248.17 ± 13	279.94 ± 27	163.5 ± 14.1
M1-CK N268L	160.59 ± 13	210.42 ± 27.1	253.1 ± 36.4	197.4 ± 7.752
Temperature (°C)	20	15	10	5
M1-CK	190.69 ± 13.6	186.7 ± 3.25	110.9 ± 4.63	89.35 ± 6.08
M1-CK N268G	181.28 ± 9.45	185 ± 12.7	109.2 ± 1.71	81.54 ± 7.62
M1CK N268D	197.56 ± 16	173.6 ± 7.41	105.2 ± 9.1	45.96 ± 1.89
M1-CK N268K	206.72 ± 3.92	178.6 ± 16.4	105.1 ± 8.19	46.91 ± 2.41
M1-CK N268L	102.51 ± 12.5	113.6 ± 4.26	77.89 ± 2.99	78.72 ± 6.49

specific activity (U)				
pH 7.7				
Temperature (°C)	40	35	30	25
M1-CK	101.84 ± 12	117.65 ± 13.8	150.94 ± 4.82	136 ± 16.8
M1-CK N268G	68.964 ± 7.6	87.723 ± 12	109.62 ± 3.18	99.19 ± 15.79
M1CK N268D	49.666 ± 3.7	182.68 ± 18.3	206.87 ± 21.8	100.2 ± 9.446
M1-CK N268K	42.438 ± 4.1	135.11 ± 9.41	172.64 ± 14.6	91.82 ± 7.701
M1-CK N268L	91.4 ± 3.7	122.01 ± 14.4	121.05 ± 3.6	133.7 ± 3.994
Temperature (°C)	20	15	10	5
M1-CK	122.56 ± 11.7	143.5 ± 7.41	95.54 ± 5.54	86.46 ± 6.53
M1-CK N268G	101.44 ± 7.48	110.6 ± 8.55	86.42 ± 2.68	70.56 ± 5.88
M1CK N268D	146.56 ± 9.76	126.9 ± 3.19	90.64 ± 3.87	45.39 ± 2.99
M1-CK N268K	140.86 ± 13.2	113.4 ± 8.17	87.15 ± 5.2	48.62 ± 3
M1-CK N268L	47.028 ± 4.24	53.24 ± 6.51	57.59 ± 5.59	74.19 ± 4.56
specific activity (U)				
pH 8.0				
Temperature (°C)	40	35	30	25
M1-CK	57.81 ± 1.3	77.57 ± 5.85	63.958 ± 1	64.55 ± 1.181
M1-CK N268G	34.676 ± 2.2	47.194 ± 3.4	46.635 ± 4.31	58.24 ± 17.19
M1CK N268D	24.17 ± 1.1	89.237 ± 5.18	95.982 ± 7.33	48.1 ± 6.383
M1-CK N268K	16.996 ± 0.9	64.94 ± 8.09	80.135 ± 4.24	34.06 ± 3.222
M1-CK N268L	52.434 ± 6.4	89.369 ± 10.9	72.096 ± 4.25	68.04 ± 2.888
Temperature (°C)	20	15	10	5
M1-CK	66.519 ± 6.62	73.08 ± 7.49	66.94 ± 13.7	26.22 ± 1.06
M1-CK N268G	52.54 ± 3.19	56.77 ± 1.39	44.18 ± 2.47	19.94 ± 1.77
M1CK N268D	90.065 ± 10.3	64.65 ± 5.26	58.14 ± 3.45	25.25 ± 2.16
M1-CK N268K	78.025 ± 2.22	55.52 ± 4.37	50.75 ± 4.2	23.05 ± 1.89
M1-CK N268L	34.592 ± 3.26	32.88 ± 5.12	22.12 ± 0.99	20.84 ± 2.85

Table 4. Mean ± S.D specific activities of M1-CK and its mutants at different temperature and pH. U was defined as 1 μmole of creatine formed per min per mg of enzyme.

specific activity (U)				
pH 7.1				
Temperature (°C)	40	35	30	25
RM-CK	292.8 ± 18	352.76 ± 23.9	241.85 ± 16.5	231.3 ± 7.795
RM-CK G268N	408.6 ± 27.1	441.3 ± 7.08	354.8 ± 38.3	311.1 ± 10.32
RM-CK G268D	376.3 ± 9.71	401.19 ± 3.72	348.68 ± 14	316.5 ± 4.993
RM-CK G268K	395 ± 3.93	407.55 ± 1.05	398.54 ± 25.9	316.2 ± 11.21
RM-CK G268L	316.2 ± 6.34	314.48 ± 4.56	275.88 ± 15.2	221.9 ± 11.11
Temperature (°C)	20	15	10	5
RM-CK	125.7 ± 5.42	83.5 ± 6.61	26 ± 3.46	16.9 ± 1.27
RM-CK G268N	190.4 ± 7.5	116 ± 15.3	65.3 ± 4.87	25.2 ± 3.86
RM-CK G268D	190.39 ± 7.64	119 ± 18.3	60 ± 0.82	30.3 ± 8.46
RM-CK G268K	200.78 ± 6.75	143 ± 16.6	68.2 ± 2.83	31.9 ± 2.27
RM-CK G268L	147.96 ± 9.83	93.8 ± 10.9	44.6 ± 4.48	22.9 ± 3.55

specific activity (U)				
pH 7.4				
Temperature (°C)	40	35	30	25
RM-CK	192.8 ± 10.2	250.67 ± 5.68	222.93 ± 20.4	210.5 ± 3.58
RM-CK G268N	308.1 ± 17.5	323.06 ± 8.98	280.9 ± 23.8	260.5 ± 15.87
RM-CK G268D	325.2 ± 15.9	296.84 ± 14.4	261.47 ± 5.59	241 ± 14.57
RM-CK G268K	304.4 ± 16.6	328.44 ± 5.24	290.65 ± 6.36	245.6 ± 11.39
RM-CK G268L	229.6 ± 5.26	224.99 ± 2.39	206.79 ± 16.7	170.9 ± 6.285
Temperature (°C)	20	15	10	5
RM-CK	129.85 ± 15	83 ± 3.98	38.7 ± 3.45	21.7 ± 2.11
RM-CK G268N	150.5 ± 6.84	139 ± 21.7	90.1 ± 7.23	31.1 ± 4.76
RM-CK G268D	158.1 ± 8.07	119 ± 12.2	72.7 ± 5.88	25.8 ± 0.58
RM-CK G268K	184.46 ± 11.1	141 ± 17.6	84.3 ± 4.15	31.4 ± 2.52
RM-CK G268L	116.56 ± 11.1	77.2 ± 8.49	54.7 ± 4.91	19.2 ± 1.62

specific activity (U)				
pH 7.7				
Temperature (°C)	40	35	30	25
RM-CK	124.4 ± 6.96	156.68 ± 11.6	153.65 ± 6.04	118.9 ± 8.981
RM-CK G268N	223.8 ± 17.4	247.47 ± 7.95	231.7 ± 19.6	159.5 ± 6.184
RM-CK G268D	224.1 ± 24.3	193.53 ± 6.94	189.63 ± 17	147 ± 7.135
RM-CK G268K	218.6 ± 9.63	223.64 ± 6.61	244.95 ± 8.73	167.5 ± 10.18
RM-CK G268L	167.9 ± 2.8	140.34 ± 0.25	130.81 ± 10.5	114.3 ± 11.72
Temperature (°C)	20	15	10	5
RM-CK	76.801 ± 5.22	58.9 ± 7.46	41.6 ± 3.87	28.9 ± 1.99
RM-CK G268N	117 ± 2.38	97 ± 8.73	82.8 ± 10.6	38.7 ± 5.57
RM-CK G268D	113.47 ± 5.89	78.4 ± 4.74	50.6 ± 5.08	37.1 ± 5.43
RM-CK G268K	136.66 ± 8.27	91.2 ± 1.71	65.3 ± 7.55	46.1 ± 3.52
RM-CK G268L	82.861 ± 5.72	53.3 ± 2.48	40.1 ± 3.57	30 ± 4.36

specific activity (U)				
pH 8.0				
Temperature (°C)	40	35	30	25
RM-CK	68.54 ± 5.06	107.79 ± 14.5	104.45 ± 10.6	82.67 ± 3.561
RM-CK G268N	142.2 ± 8.99	148.5 ± 7.71	114 ± 11.2	108 ± 10.19
RM-CK G268D	113.2 ± 4.74	108.73 ± 3.47	111.72 ± 10.8	99.26 ± 8.758
RM-CK G268K	123.2 ± 6.94	122.25 ± 5.8	140.44 ± 11.2	120.3 ± 14.95
RM-CK G268L	83.72 ± 4.47	77.193 ± 0.69	73.487 ± 3.56	61.6 ± 6.184
Temperature (°C)	20	15	10	5
RM-CK	50.638 ± 4.84	29.1 ± 7.52	29 ± 3.14	27.1 ± 2.69
RM-CK G268N	69.85 ± 6.45	51 ± 2.75	66.2 ± 7.61	29.5 ± 3.61
RM-CK G268D	57.128 ± 3.22	45 ± 1.56	34.5 ± 2.19	24.9 ± 1.03
RM-CK G268K	72.075 ± 6.12	50.4 ± 1.57	45.3 ± 1.98	30.4 ± 2.72
RM-CK G268L	40.981 ± 2.4	31.2 ± 3.34	20.6 ± 1.28	18.5 ± 3.68

Table 5. Mean ± S.D specific activities of RM-CK and its mutants at different temperature and pH. U was defined as 1 μmole of creatine formed per min per mg of enzyme

K_m^{ADP} (mM)								
pH 7.1								
Temperature (oC)	35		25		15		5	
M1-CK	1.41 ±	0.12	0.88 ±	0.1	1.02 ±	0.112	0.7 ±	0.0679
M1-CK N268G	1.21 ±	0.03	1.35 ±	0.18	0.9 ±	0.037	0.29 ±	0.0303
M1-CK N268D	1.23 ±	0.05	0.8 ±	0.09	0.98 ±	0.12	0.27 ±	0.0216
M1-CK N268K	1.51 ±	0.05	1.74 ±	0.11	1.34 ±	0.151	0.38 ±	0.0658
M1-CK N268L	1.54 ±	0.05	0.49 ±	0.02	0.55 ±	0.072	0.43 ±	0.0112
pH 8.0								
Temperature (°C)	35		25		15		5	
M1-CK	0.2 ±	0.01	0.3 ±	0.04	0.26 ±	0.037	0.45 ±	0.016
M1-CK N268G	0.45 ±	0.01	0.22 ±	0.01	0.44 ±	0.026	0.29 ±	0.0401
M1-CK N268D	0.35 ±	0.03	0.26 ±	0.02	1.16 ±	0.123	0.27 ±	0.0221
M1-CK N268K	0.49 ±	0.07	0.28 ±	0.02	0.49 ±	0.018	0.3 ±	0.0274
M1-CK N268L	0.27 ±	0.03	0.22 ±	0.01	0.31 ±	0.016	0.26 ±	0.0214
K_m^{PCr} (mM)								
pH 7.1								
Temperature (°C)	35		25		15		5	
M1-CK	6.75 ±	0.6	11.3 ±	1	22.8 ±	3.331	83.6 ±	4.5527
M1-CK N268G	7.1 ±	0.62	29.8 ±	3.63	55.3 ±	8.562	132 ±	16.169
M1-CK N268D	6.45 ±	0.38	23.2 ±	2.27	48.3 ±	3.335	100 ±	6.3506
M1-CK N268K	8.59 ±	0.53	20.5 ±	1.09	44.2 ±	6.54	95.4 ±	12.95
M1-CK N268L	15.2 ±	1.26	24.6 ±	2	44.7 ±	2.905	110 ±	9.3948
pH 8.0								
Temperature (oC)	35		25		15		5	
M1-CK	25.7 ±	1.19	25.3 ±	3.73	18 ±	2.651	14.6 ±	0.8607
M1-CK N268G	22.5 ±	0.87	11.1 ±	1.35	10.3 ±	1.497	21.7 ±	3.1897
M1-CK N268D	23.7 ±	2.28	10.2 ±	0.15	10.8 ±	1.482	22.6 ±	2.0667
M1-CK N268K	33.7 ±	2.61	37.1 ±	5.48	12.4 ±	0.391	13.1 ±	0.4827
M1-CK N268L	23.2 ±	2.65	13.7 ±	1.16	16 ±	2.039	21.7 ±	2.9257

Table 6. Mean ± S.D K_m values of M1-CK and its mutants at 35, 25, 15, 5 °C at pH 7.1, 8.0.

$K_{cat}^{ADP} \text{ (sec}^{-1}\text{)}$								
pH 7.1								
Temperature (oC)	35		25		15		5	
M1-CK	419 ±	40.3	266 ±	18.4	152 ±	19.82	57.5 ±	2.1291
M1-CK N268G	475 ±	19	475 ±	71.4	195 ±	23.44	39.1 ±	1.3447
M1-CK N268D	433 ±	62.3	564 ±	6.92	196 ±	19.88	35.9 ±	2.2202
M1-CK N268K	687 ±	65.8	720 ±	53.6	283 ±	19.38	66.3 ±	8.6215
M1-CK N268L	536 ±	31.5	243 ±	9.06	144 ±	17.53	70.5 ±	6.9784
pH 8.0								
Temperature (oC)	35		25		15		5	
M1-CK	36.7 ±	1.56	58.2 ±	7.68	40.7 ±	4.444	34.1 ±	0.8261
M1-CK N268G	62.4 ±	4.21	45.3 ±	7.73	44.7 ±	6.1	19.4 ±	2.8613
M1-CK N268D	101 ±	12.4	53.1 ±	3.89	88.5 ±	10.8	19.1 ±	2.029
M1-CK N268K	132 ±	4.25	70.3 ±	6.76	77.1 ±	8.478	26.5 ±	2.848
M1-CK N268L	57.4 ±	8.51	101 ±	13.7	44.7 ±	5.76	29.5 ±	2.7293
$K_{cat}^{PCr} \text{ (sec}^{-1}\text{)}$								
pH 7.1								
Temperature (oC)	35		25		15		5	
M1-CK	258 ±	29.4	185 ±	23.8	96.2 ±	11.46	59.8 ±	7.7761
M1-CK N268G	233 ±	34	348 ±	54.2	136 ±	18.36	75.1 ±	11.568
M1-CK N268D	334 ±	38	303 ±	33.6	136 ±	8.31	33.4 ±	4.9646
M1-CK N268K	387 ±	38.5	392 ±	27.7	151 ±	16.96	56.9 ±	10.057
M1-CK N268L	485 ±	38.8	199 ±	12.9	105 ±	9.532	65.9 ±	5.2735
pH 8.0								
Temperature (oC)	35		25		15		5	
M1-CK	56.9 ±	6.45	68.5 ±	9.6	62.7 ±	8.203	55.5 ±	0.2716
M1-CK N268G	86.4 ±	9.76	79.5 ±	9.02	70 ±	3.678	31.1 ±	5.647
M1-CK N268D	134 ±	7.13	62.5 ±	6.23	71 ±	7.824	27.1 ±	1.57
M1-CK N268K	96.1 ±	17.5	156 ±	17.4	99.5 ±	8.267	31.4 ±	0.5261
M1-CK N268L	62.8 ±	4.3	169 ±	13.7	65.3 ±	6.391	53.2 ±	7.6713

Table 7. Mean ± S.D K_{cat} values of M1-CK and its mutants at 35, 25, 15, 5 °C at pH 7.1, 8.0

K_m^{ADP} (mM)								
pH 7.1								
Temperature (°C)	35		25		15		5	
RM-CK	1.43 ±	0.11	0.78 ±	0.11	0.37 ±	0.038	0.17 ±	0.025
RM-CK G268N	1.36 ±	0.14	0.53 ±	0.03	0.33 ±	0.019	0.14 ±	0.017
RM-CK G268D	0.77 ±	0.16	0.54 ±	0.08	0.23 ±	0.028	0.12 ±	0.015
RM-CK G268K	1.53 ±	0.18	0.65 ±	0.07	0.37 ±	0.042	0.23 ±	0.015
RM-CK G268L	1.17 ±	0.06	0.48 ±	0.07	0.29 ±	0.034	0.14 ±	0.011
pH 8.0								
Temperature (°C)	35		25		15		5	
RM-CK	0.31 ±	0.05	0.14 ±	0.01	0.12 ±	0.016	0.11 ±	0.002
RM-CK G268N	0.21 ±	0.03	0.14 ±	0.02	0.11 ±	0.011	0.08 ±	0.004
RM-CK G268D	0.41 ±	0.06	0.2 ±	0.04	0.1 ±	0.016	0.12 ±	0.014
RM-CK G268K	0.31 ±	0.03	0.32 ±	0.04	0.23 ±	0.028	0.11 ±	0.015
RM-CK G268L	0.22 ±	0.03	0.18 ±	0.01	0.16 ±	0.015	0.06 ±	0.005
K_m^{PCr} (mM)								
pH 7.1								
Temperature (°C)	35		25		15		5	
RM-CK	7.09 ±	0.41	10.1 ±	1.38	20.4 ±	2.304	64.8 ±	3.949
RM-CK G268N	5.16 ±	0.75	8.06 ±	0.38	33.9 ±	4.095	28.9 ±	2.773
RM-CK G268D	3.48 ±	0.46	23.7 ±	1.65	44.2 ±	0.518	109 ±	12.037
RM-CK G268K	3.68 ±	0.34	12.3 ±	1.45	31.6 ±	3.276	68.6 ±	10.438
RM-CK G268L	6.57 ±	0.9	15.9 ±	1.57	41.3 ±	0.773	116 ±	18.922
pH 8.0								
Temperature (°C)	35		25		15		5	
RM-CK	11.3 ±	1.63	8.73 ±	0.5	7.42 ±	0.807	5.91 ±	0.666
RM-CK G268N	9.75 ±	0.35	8.04 ±	1.08	6.31 ±	0.471	4.27 ±	0.432
RM-CK G268D	20.1 ±	1.14	14.7 ±	2.79	7.82 ±	0.44	1.4 ±	0.152
RM-CK G268K	10.8 ±	0.67	8.14 ±	0.63	6.91 ±	0.879	9.51 ±	0.631
RM-CK G268L	9.29 ±	1.08	7.13 ±	0.35	5.97 ±	0.538	8.93 ±	1.677

Table 8. Mean ± S.D K_m values of RM-CK and its mutants at 35, 25, 15, 5 °C at pH 7.1, 8.0.

K_{cat}^{ADP} (sec ⁻¹)								
pH 7.1								
Temperature (oC)	35		25		15		5	
RM-CK	473 ±	31.9	264 ±	15.9	72.4 ±	9.536	20.1 ±	2.501
RM-CK G268N	443 ±	44.6	204 ±	9.67	63.3 ±	5.886	16.8 ±	2.457
RM-CK G268D	271 ±	67.5	206 ±	37.2	48.3 ±	3.975	21.1 ±	2.309
RM-CK G268K	598 ±	33	284 ±	20.1	97.5 ±	8.354	31.6 ±	0.611
RM-CK G268L	281 ±	5.17	248 ±	10.6	65 ±	15	23.6 ±	2.603
pH 8.0								
Temperature (oC)	35		25		15		5	
RM-CK	78 ±	11.5	49.6 ±	7.38	22 ±	3.016	10.6 ±	0.819
RM-CK G268N	73.1 ±	1.23	48.8 ±	4.06	22.3 ±	2.607	9.44 ±	0.811
RM-CK G268D	95.3 ±	12.7	32.1 ±	2.79	14.7 ±	1.868	12.2 ±	1.58
RM-CK G268K	100 ±	6.61	81.3 ±	5.19	52.7 ±	8.878	19.6 ±	3.931
RM-CK G268L	76.7 ±	5.06	40.6 ±	13	28.6 ±	1.199	9.71 ±	4.517
K_{cat}^{PCr} (sec ⁻¹)								
pH 7.1								
Temperature (oC)	35		25		15		5	
RM-CK	398 ±	34	196 ±	23.8	59.7 ±	5.475	24.2 ±	0.238
RM-CK G268N	287 ±	36.9	149 ±	5.03	64.2 ±	5.849	16.7 ±	1.388
RM-CK G268D	287 ±	35.4	194 ±	20.1	46.3 ±	2.685	19 ±	6.903
RM-CK G268K	353 ±	22.1	266 ±	39.6	92.6 ±	14.91	43.9 ±	5.289
RM-CK G268L	365 ±	96.3	248 ±	35	82.9 ±	10.66	32.3 ±	3.281
pH 8.0								
Temperature (oC)	35		25		15		5	
RM-CK	75.2 ±	9.26	67.5 ±	3.5	39.4 ±	4.615	22.6 ±	3.113
RM-CK G268N	87 ±	12.5	67.4 ±	5.63	27.8 ±	2.516	17.6 ±	1.723
RM-CK G268D	155 ±	13.5	76.3 ±	8.79	23.6 ±	3.327	11.4 ±	0.266
RM-CK G268K	149 ±	18.9	115 ±	18.3	48.1 ±	7.234	33.3 ±	1.638
RM-CK G268L	85.8 ±	8.56	87.3 ±	11.4	44.6 ±	4.01	21.6 ±	5.216

Table 9. Mean ± S.D K_{cat} values of RM-CK and its mutants at 35, 25, 15, 5 °C at pH 7.1, 8.0.

solvent accessible surface (SAS) Å ²					
	RM-CK	RM-CK G268N	RM-CK G268D	RM-CK G268K	RM-CK G268L
Lys ¹	91.4	100.3	92	99.9	72.3
Tyr ¹⁴⁰	39.3	46	35.9	40.8	37.3
Thr ¹⁴¹	9.3	13.1	8	12.1	12.5
His ¹⁴⁵	55	55.3	58	64	62.2
Cys ¹⁴⁶	5	4.3	5.4	6.9	5.9
Ser ¹⁴⁷	53	52.2	57.5	58.8	58
Arg ¹⁴⁸	126.4	122.2	128.2	125.1	122
Glu ¹⁵⁰	45.4	40.9	51.7	44.3	38.8
Arg ¹⁵¹	4.6	4.5	4.3	3.1	6.2
Arg ¹⁵²	122.5	119	115.6	118.6	113.9
Gln ²⁵⁸	79.6	81.9	100.9	75.6	89.7
Lys ²⁵⁹	69.4	76	60.3	62.6	42.9
Glu ²⁶¹	51	39.5	31.9	43.5	50.5
Glu ²⁶²	88.2	75.3	88.3	68.7	109.1
Lys ²⁶⁵	103.5	129.6	109.7	96.6	106.6
Lys ²⁶⁶	172.2	152.1	171	155.8	160.1
His ²⁶⁹	41	58.6	48.1	26.8	45

solvent accessible surface (SAS) Å ²					
	M1-CK	M1-CK N268G	M1-CK N268D	M1-CK N268K	M1-CK N268L
Asp ⁷⁸	7.1	6.7	2.5	5.5	5.8
Glu ⁷⁹	57.8	36.8	64.9	42.4	49
Glu ⁸⁰	44	51.2	55.5	68.4	63.2
Ser ⁸¹	0.5	0.5	0	0.9	0.9
Tyr ⁸²	10.9	9.9	10.8	13.2	11.3
Glu ⁸³	142.8	124.9	127.1	133.9	136.3

Table 10. The solvent accessible surface (SAS) of hydrophilic residues nearby residue 268

	C α distance (Å)				
	RM-CK	RM-CK G268N	RM-CK G268D	RM-CK G268K	RM-CK G268L
Asp ⁷⁸	17.08	17.71	17.11	17.1	17.05
Glu ⁷⁹	15.6	16.32	15.69	15.64	15.66
Glu ⁸⁰	17.81	18.81	17.88	17.84	17.81
Ser ⁸¹	20.88	21.75	20.92	20.89	20.86
Tyr ⁸²	21.7	22.1	21.69	21.62	21.65
Thr ⁸³	21.87	22.12	21.85	21.78	21.82
Glu ¹⁶⁰	14.13	12.91	14.21	14.21	14.33
Asn ¹⁶³	18.23	16.07	18.23	18.19	18.34
Ser ¹⁶⁴	17.14	15.58	16.99	17.06	17.16
Thr ¹⁶⁶	21.93	20.03	21.87	21.91	21.9
Glu ¹⁶⁸	25.87	24.06	25.7	25.72	25.68
Lys ¹⁷⁰	23.44	21.79	23.36	23.39	23.49
Lys ¹⁷²	23.85	23.01	24.04	23.89	24.04
Lys ²⁵⁹	14.87	13.43	14.58	14.63	14.67
Glu ²⁶¹	10.78	9.45	10.43	10.43	10.43
Glu ²⁶²	9.92	8.03	9.57	9.69	9.66
Lys ²⁶⁵	5.34	4.2	4.87	5.06	5.04
Lys ²⁶⁶	5.51	4	5.19	5.23	5.29
His ²⁶⁹	3.79	3.73	3.77	3.76	3.76

Table 11. C α distances of residue 268 to nearby hydrophilic residues of RM-CK and its mutants.

	C α distance (Å)				
	M1-CK	M1-CK N268G	M1-CK N268D	M1-CK N268K	M1-CK N268L
Glu ¹⁶⁰	14.19	14.1	14.1	14.22	14.14
Asn ¹⁶³	18.2	18.13	18.19	18.22	18.28
Ser ¹⁶⁴	16.95	17.05	16.99	17.01	17
Asp ¹⁶⁶	21.87	22.04	21.85	21.86	21.95
Glu ¹⁶⁸	25.68	25.8	25.72	25.68	25.85
Lys ¹⁷⁰	23.39	23.51	23.32	23.34	23.4
Lys ¹⁷²	23.9	23.92	23.94	23.96	23.96
Gln ²⁵⁸	15.08	15.2	15.09	14.98	15.17
Arg ²⁵⁹	15.08	15.2	15.09	14.98	15.17
Glu ²⁶¹	14.66	14.76	14.67	14.56	14.72
Glu ²⁶²	10.48	10.57	10.51	10.42	10.57
Lys ²⁶⁵	9.79	9.8	9.71	9.57	9.89
Lys ²⁶⁶	5.1	5.16	5.1	4.93	5.15
His ²⁶⁷	5.28	5.28	5.25	5.17	5.24
His ²⁶⁹	3.8	3.76	3.78	3.82	3.8

Table 12. C α distances of residue 268 to nearby hydrophilic residues of M1-CK and its mutants.

	C α distance (Å)				
	RM-CK	RM-CK G268N	RM-CK G268D	RM-CK G268K	RM-CK G268L
Asn ⁶³	14.61	14.91	14.67	14.61	14.6
Pro ⁶⁴	16.73	16.11	16.8	16.71	16.84
Gly ⁶⁵	16.37	16.27	16.25	16.15	16.23
His ⁶⁶	18.67	18.28	18.73	18.58	18.76
Pro ⁶⁷	20.16	19.86	20.06	19.96	20.12
Phe ⁶⁸	20.14	20.31	20.04	19.88	20.06
Ile ⁶⁹	16.48	16.58	16.36	16.2	16.39
Met ⁷⁰	13.61	13.98	13.69	13.55	13.69
Thr ⁷¹	10.25	10.43	10.28	10.2	10.33
*Val ⁷²	7.64	7.64	7.66	7.58	7.67
Gly ⁷³	8.82	8.73	8.83	8.79	8.85
Cys ⁷⁴	7.03	6.71	7.01	6.99	7.01
*Arg ¹³⁰	21.44	21.29	21.34	21.25	21.27
Val ¹³¹	18.78	18.79	18.67	18.6	18.63
*Arg ¹³²	15.54	15.43	15.44	15.33	15.37
Thr ¹³³	12.84	12.48	12.75	12.78	12.73
Gly ¹³⁴	10.55	10.96	10.5	10.44	10.45
Asn ²³⁰	14.41	14.41	14.29	14.13	14.26
Glu ²³¹	12.74	12.77	12.75	12.77	12.69
*Glu ²³²	8.96	9.09	8.98	8.99	8.92
Asp ²³³	8.81	8.95	8.85	8.83	8.84
His ²³⁴	12.24	12.17	12.2	12.16	12.18
Leu ²³⁵	13.4	13.24	13.37	13.34	13.34
*Arg ²³⁶	13.19	12.99	13.15	13.08	13.1
Pro ²⁸⁴	3.84	3.87	3.76	3.77	3.76
Ser ²⁸⁵	5.51	5.39	5.51	5.55	5.48
Asn ²⁸⁶	5.74	5.61	5.62	5.62	5.77
Arg ³¹⁶	21.02	21.7	21.1	21.14	21.23
Leu ³¹⁷	20.25	20.46	20.44	20.44	20.44
Gln ³¹⁸	19.13	19.72	19.16	19.17	19.33
Lys ³¹⁹	20.26	20.9	20.42	20.38	20.53
*Arg ³²⁰	19.77	19.73	19.76	19.75	19.7

Table 13. C α distances of residue 283 to active site residues of RM-CK and its mutants. Boundary residues of active site are indicated by asterisk.

	C α distance (Å)				
	M1-CK	M1-CK N268G	M1-CK N268D	M1-CK N268K	M1-CK N268L
Asn ⁶³	14.74	14.74	14.7	14.65	14.68
Pro ⁶⁴	16.79	16.8	16.74	16.74	16.76
Gly ⁶⁵	16.17	16.19	16.16	16.17	16.16
His ⁶⁶	18.56	18.67	18.58	18.69	18.62
Pro ⁶⁷	19.98	20.06	19.97	20.01	20.01
Phe ⁶⁸	19.88	19.97	19.96	19.9	19.95
Ile ⁶⁹	16.3	16.31	16.34	16.29	16.33
Met ⁷⁰	13.59	13.69	13.62	13.66	13.65
Thr ⁷¹	10.23	10.27	10.24	10.22	10.27
*Val ⁷²	7.61	7.58	7.59	7.6	7.61
Gly ⁷³	8.81	8.82	8.8	8.8	8.8
Cys ⁷⁴	7.03	7.02	6.99	7.01	7
*Arg ¹³⁰	21.26	21.36	21.29	21.21	21.25
Val ¹³¹	18.65	18.77	18.68	18.6	18.64
*Arg ¹³²	15.39	15.58	15.49	15.43	15.39
Thr ¹³³	12.76	12.86	12.76	12.7	12.75
Gly ¹³⁴	10.49	10.57	10.52	10.46	10.51
Asn ²³⁰	14.26	14.3	14.26	14.31	14.34
Glu ²³¹	12.75	12.74	12.75	12.73	12.77
*Glu ²³²	8.97	8.97	8.97	8.97	8.99
Asp ²³³	8.81	8.87	8.85	8.82	8.84
His ²³⁴	12.15	12.22	12.19	12.17	12.19
Leu ²³⁵	13.37	13.42	13.4	13.36	13.39
*Arg ²³⁶	13.1	13.2	13.19	13.12	13.17
Pro ²⁸⁴	3.76	3.74	3.76	3.77	3.76
Ser ²⁸⁵	5.47	5.44	5.52	5.42	5.51
Asn ²⁸⁶	5.59	5.65	5.58	5.73	5.61
Arg ³¹⁶	20.91	21.18	20.94	21.04	20.96
Leu ³¹⁷	20.31	20.48	20.26	20.26	20.25
Gln ³¹⁸	18.86	19.2	19	19	18.96
Lys ³¹⁹	20.28	20.55	20.31	20.29	20.23
*Arg ³²⁰	19.58	19.66	19.74	19.52	19.58

Table 14. C α distances of residue 283 to active site residues of M1-CK and its mutants.

Boundary residues of active site are indicated by asterisk.

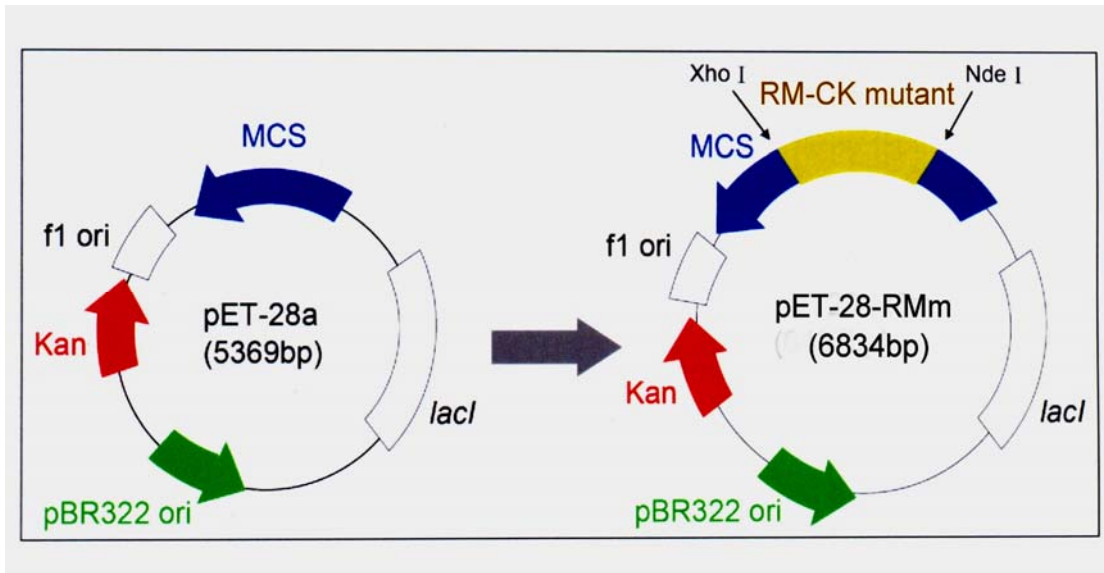


Figure 1. Construction of RM-CK and M1-CK mutants with pET28a (+). The vector was 5369bp and the insert was 1146 bp, total size of the plasmid was 6834 bp.

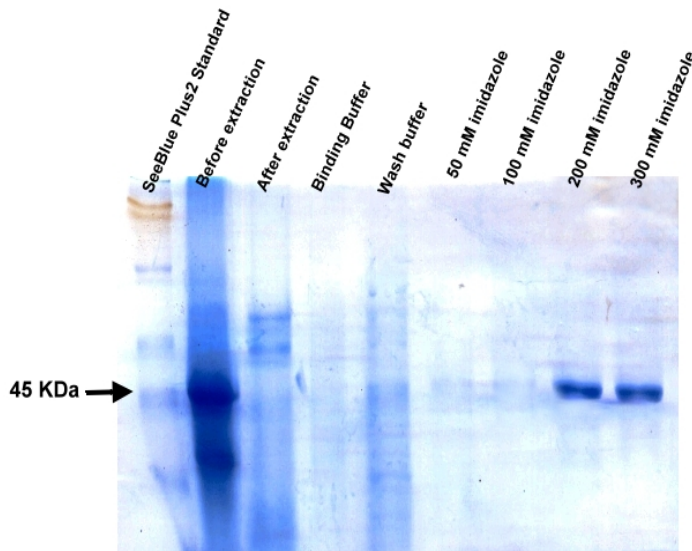


Figure 2. Protein extracted products analysis in SDS-PAGE. The protein was eluted in 200mM and 300 mM imidazole elution buffer.

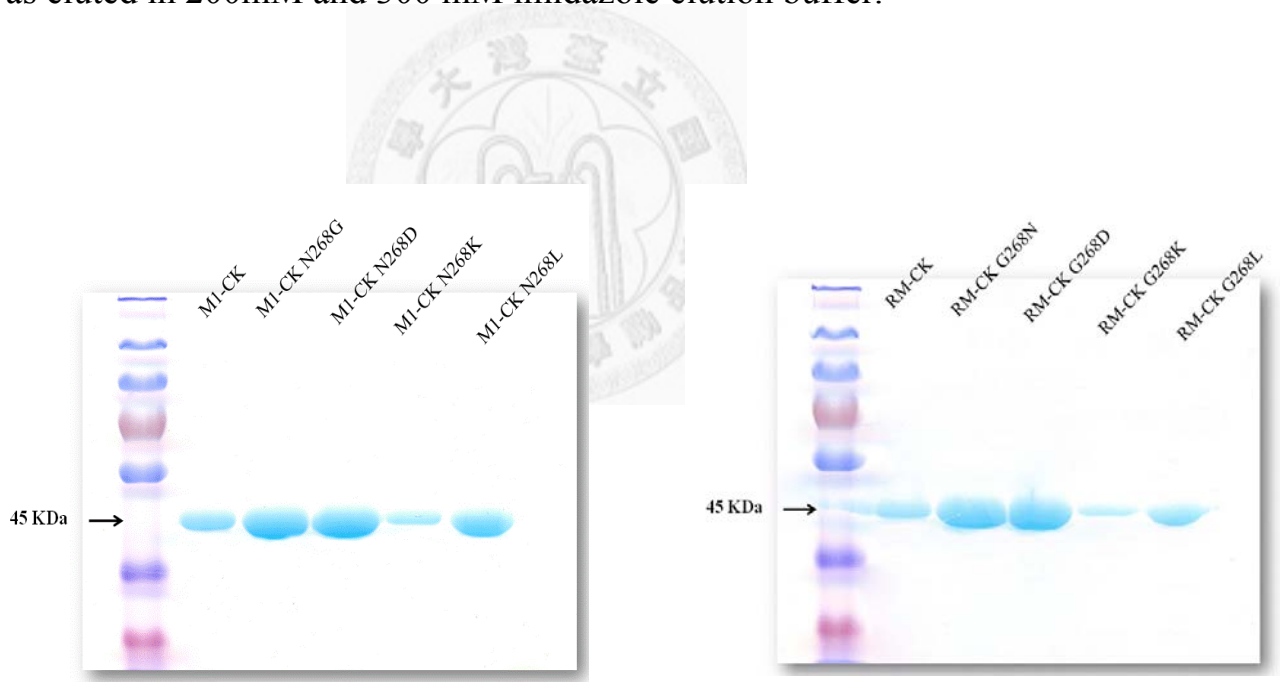


Figure 3. Wild type and mutant proteins analysed by SDS-PAGE. All the mutant proteins are of the same size as the wild-type protein, about 45 kDa

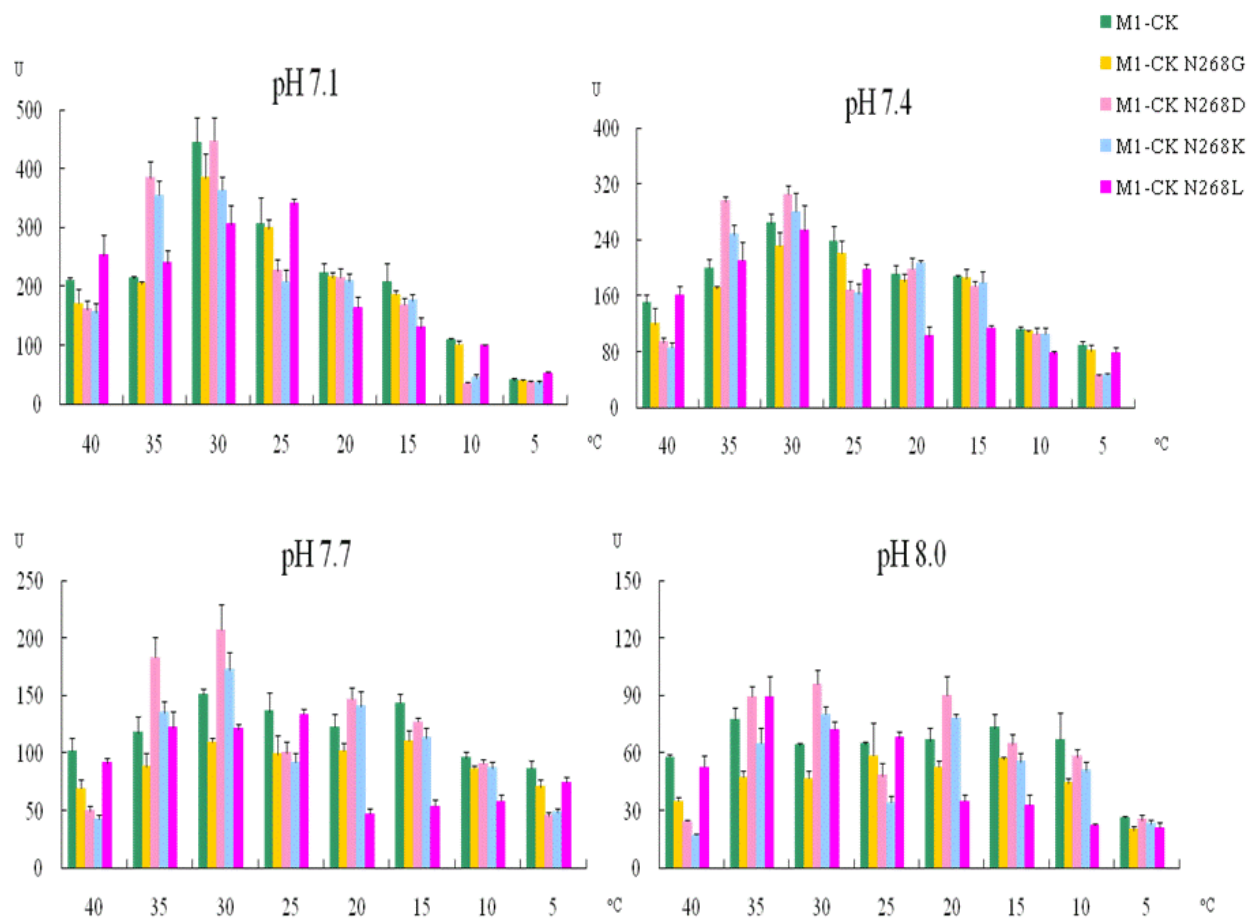


Figure 4. Mean \pm S.D specific activities of M1-CK (green), M1-CK N268G (yellow), M1-CK N268D (pink), M1-CK N268K (light blue) and M1-CK N268L (magenta) at different temperature and pH. U was defined as 1 μ mole of creatine formed per min per mg of enzyme.

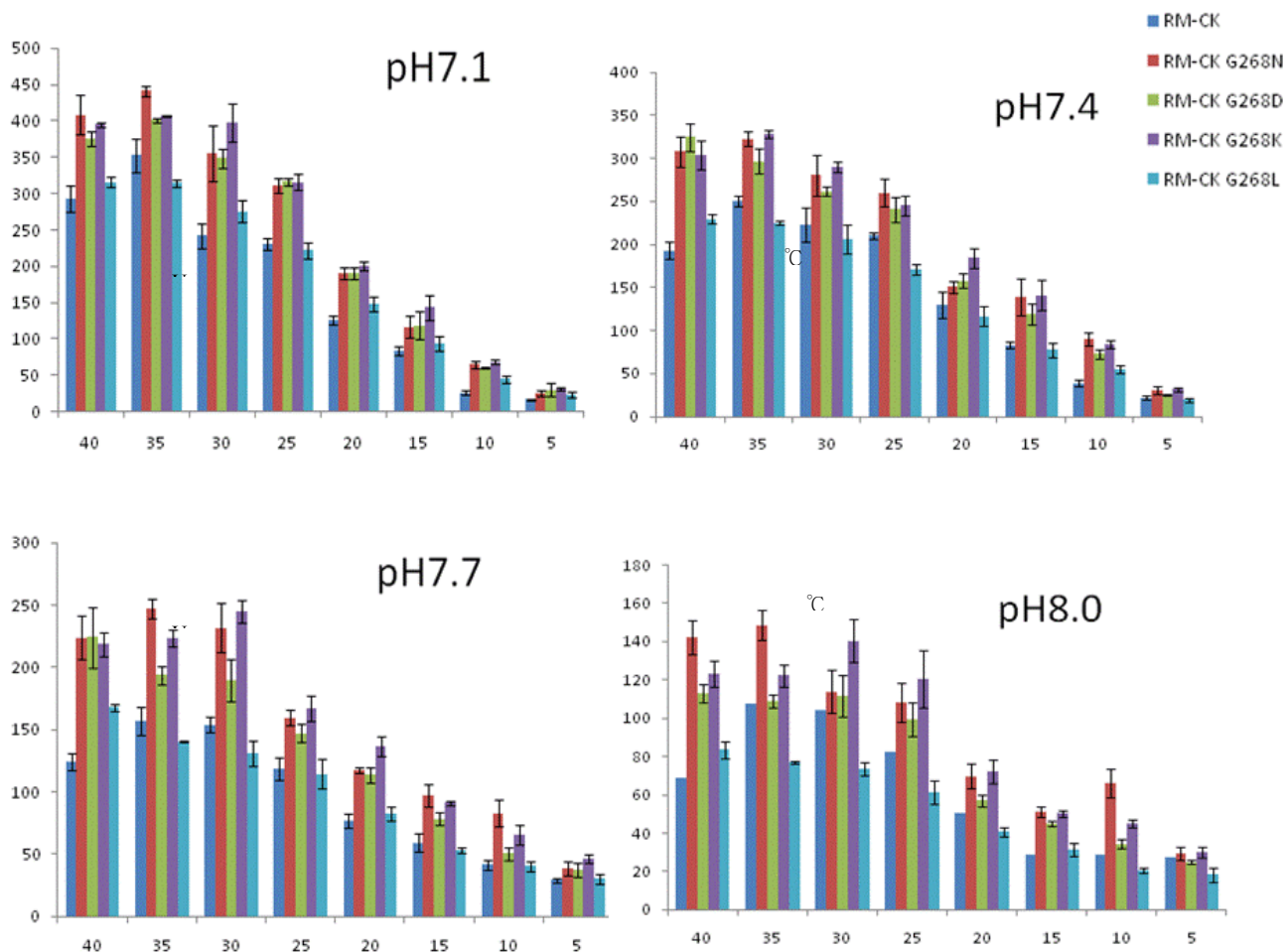


Figure 5. Mean \pm S.D specific activities of RM-CK (blue), RM-CK G268N (red), RM-CK G268D (green), RM-CK G268K (purple) and RM-CK G268L (cyan) at different temperature and pH. U was defined as 1 μ mole of creatine formed per min per mg of enzyme.

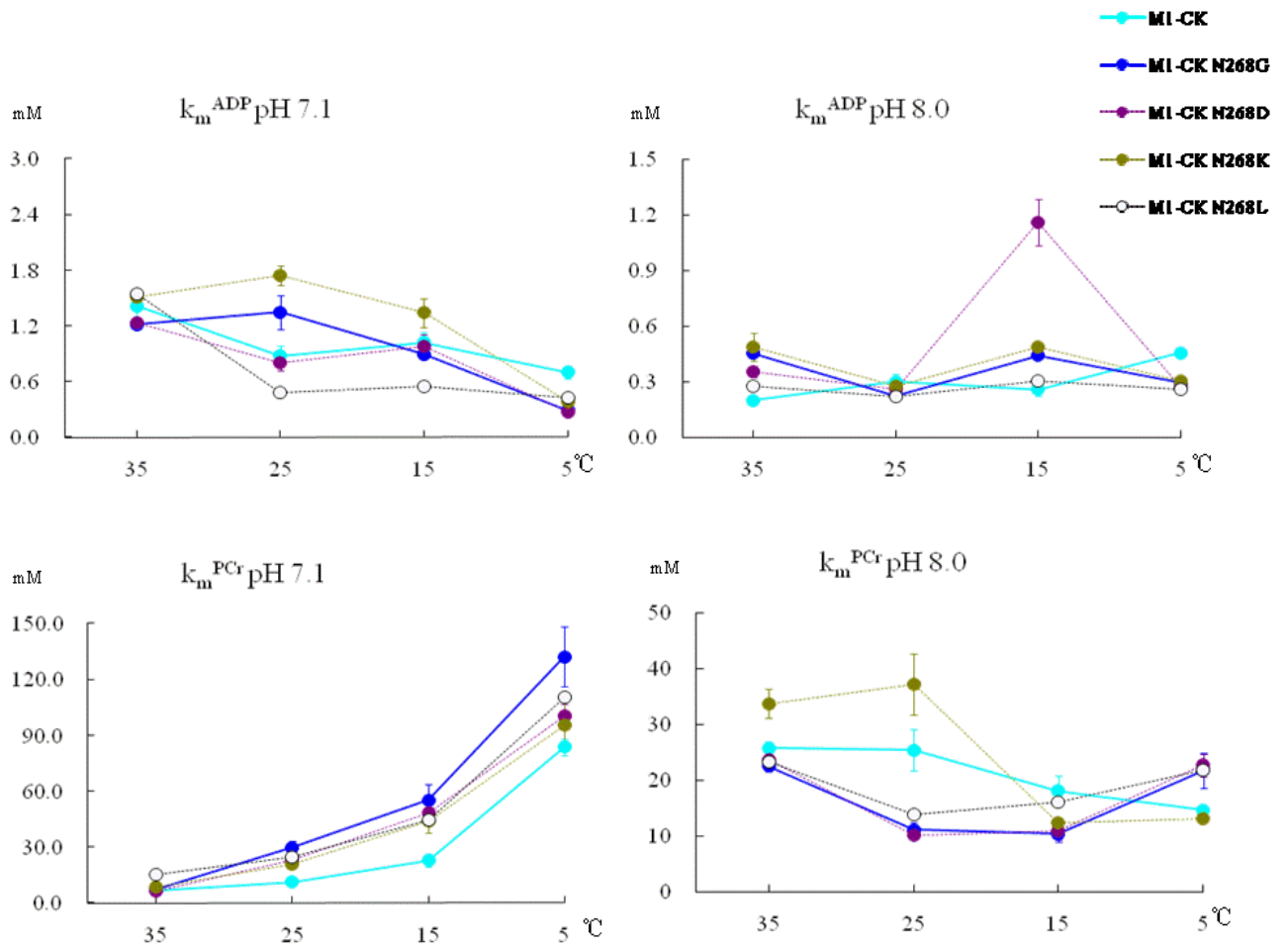


Figure 6. Mean \pm S.D K_m of M1-CK (cyan), M1-CK N268G (blue), M1-CK N268D (purple), M1-CK N268K (green) and M1-CK N268L (white) at different temperature at pH 7.1 and 8.0.

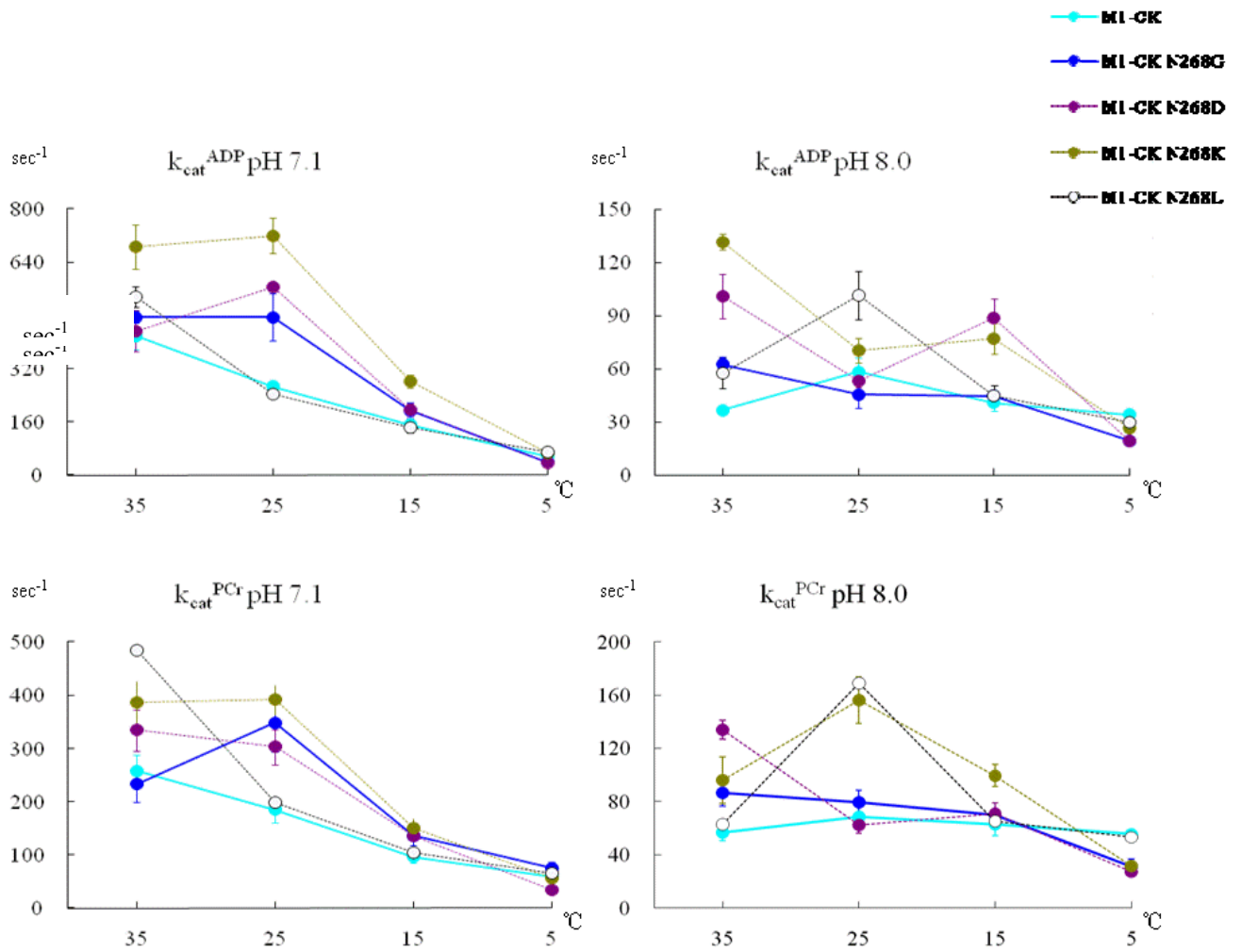


Figure 7. Mean \pm S.D k_{cat} of M1-CK (cyan), M1-CK N268G (blue), M1-CK N268D (purple), M1-CK N268K (green) and M1-CK N268L (white) at different temperature at pH 7.1 and 8.0.

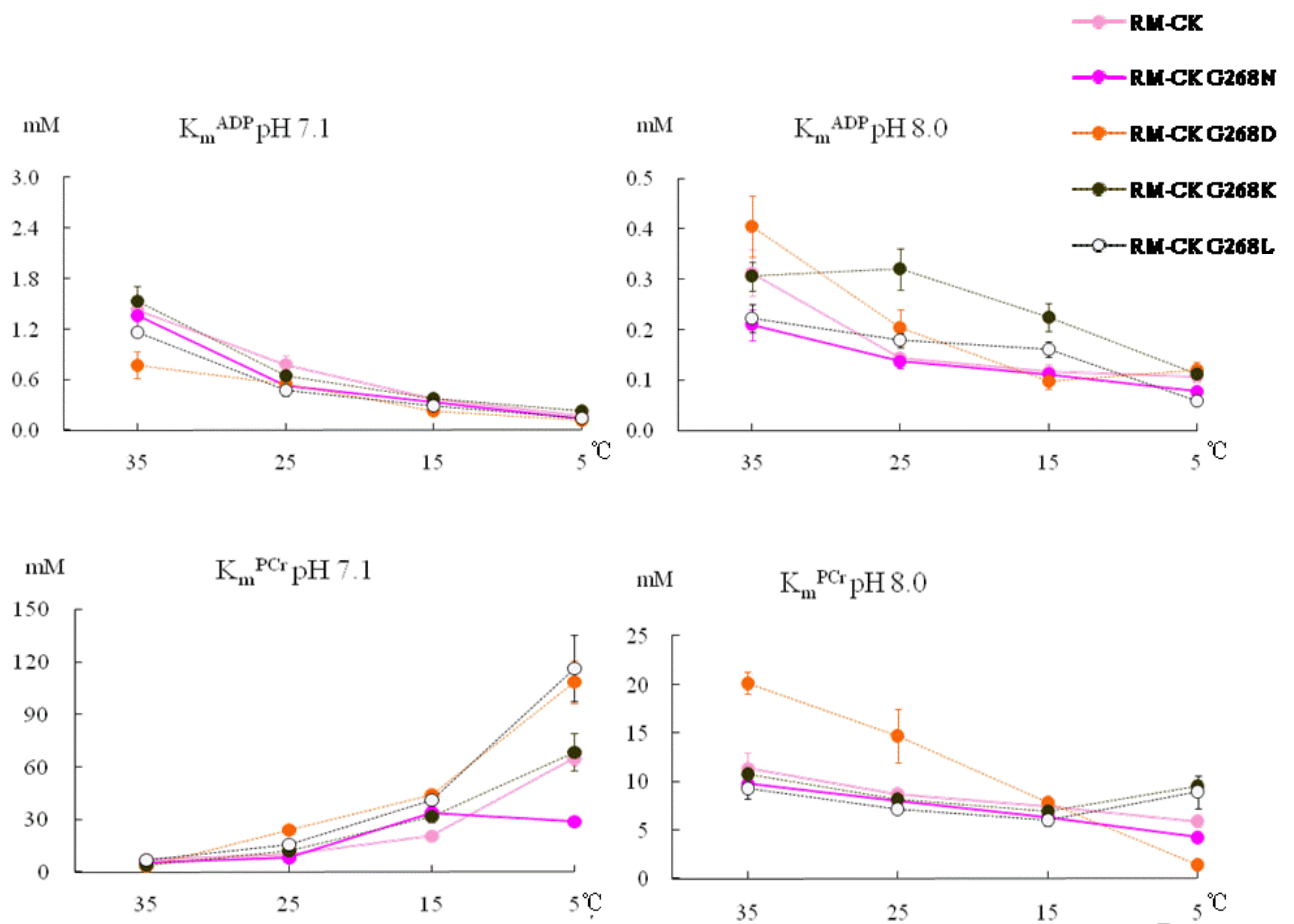


Figure 8. Mean \pm S.D K_m of RM-CK (pink), RM-CK G268N (magenta), RM-CK G268D (orange), RM-CK G268K (dark green) and RM-CK G268L (white) at different temperature and pH.

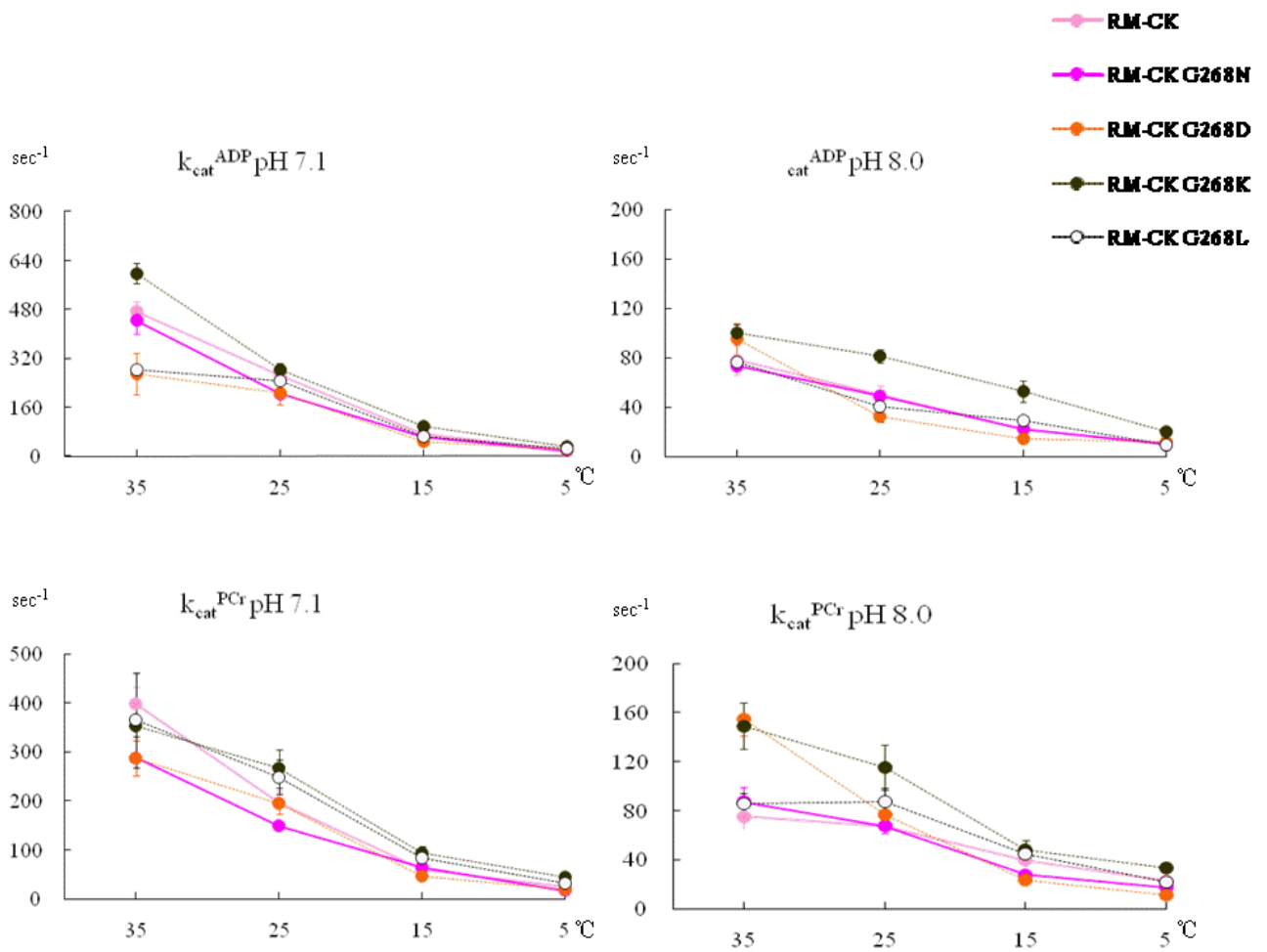


Figure 9. Mean \pm S.D k_{cat} of RM-CK (pink), RM-CK G268N (magenta), RM-CK G268D (orange), RM-CK G268K (dark green) and RM-CK G268L (white) at different temperature and pH.

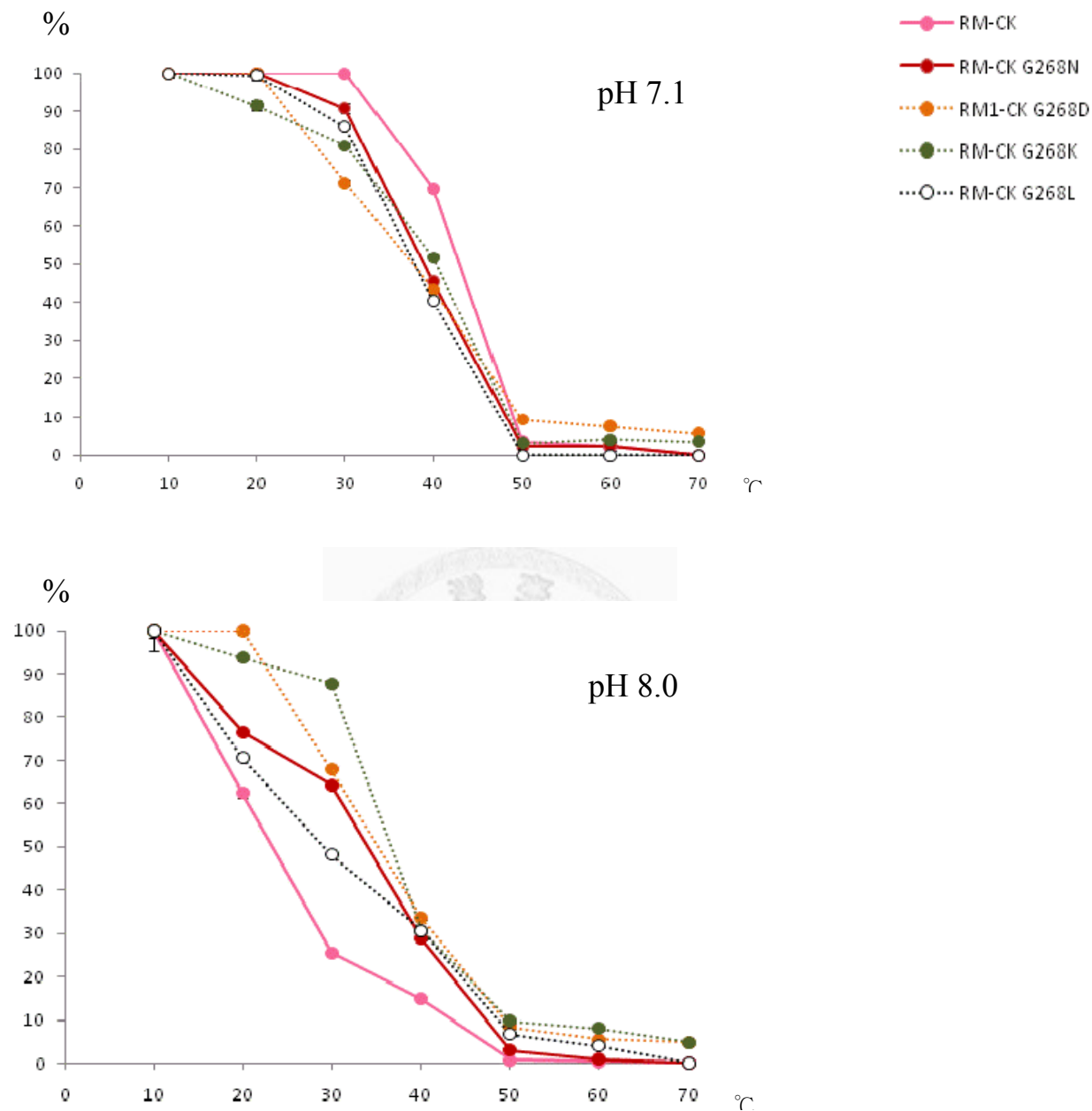


Figure 10. Thermal stability of RM-CK (pink), RM-CK G268N (red), RM-CK G268D (orange), RM-CK G268K (green) and RM-CK G268L (white) at pH 7.1 and 8.0 .

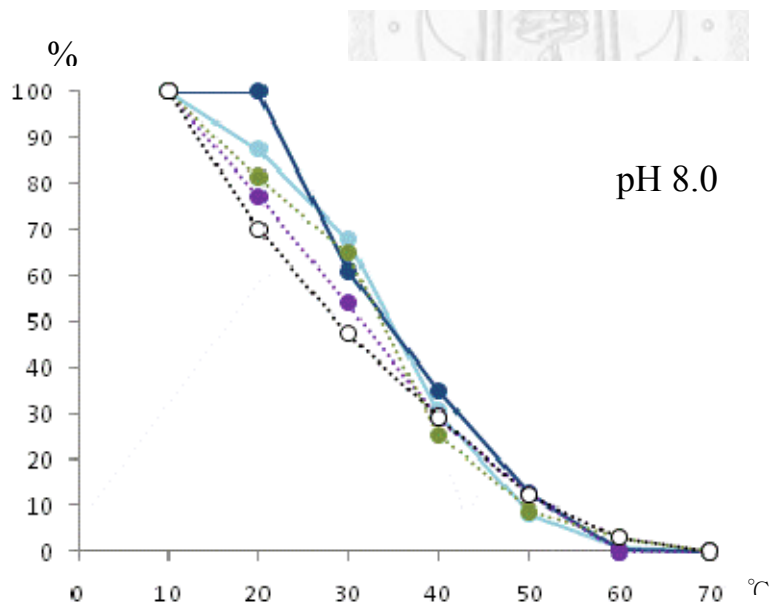
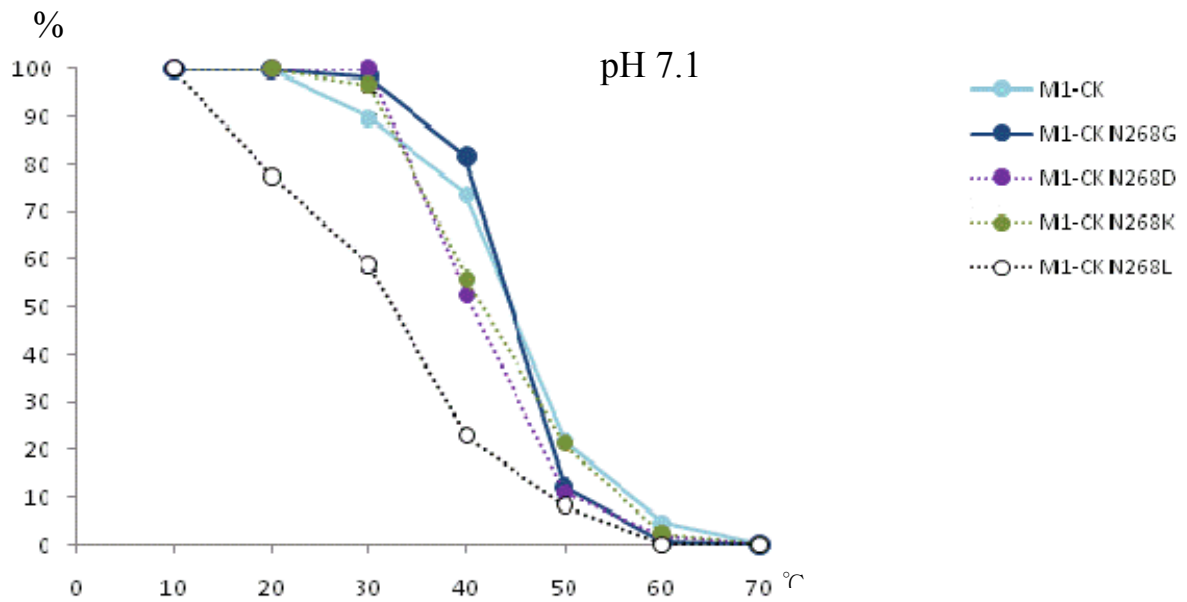


Figure 11. Thermal stability of M1-CK (cyan), M1-CK N268G (blue), M1-CK N268D (purple), M1-CK N268K (green) and M1-CK N268L (white) at pH 7.1 and 8.0 .

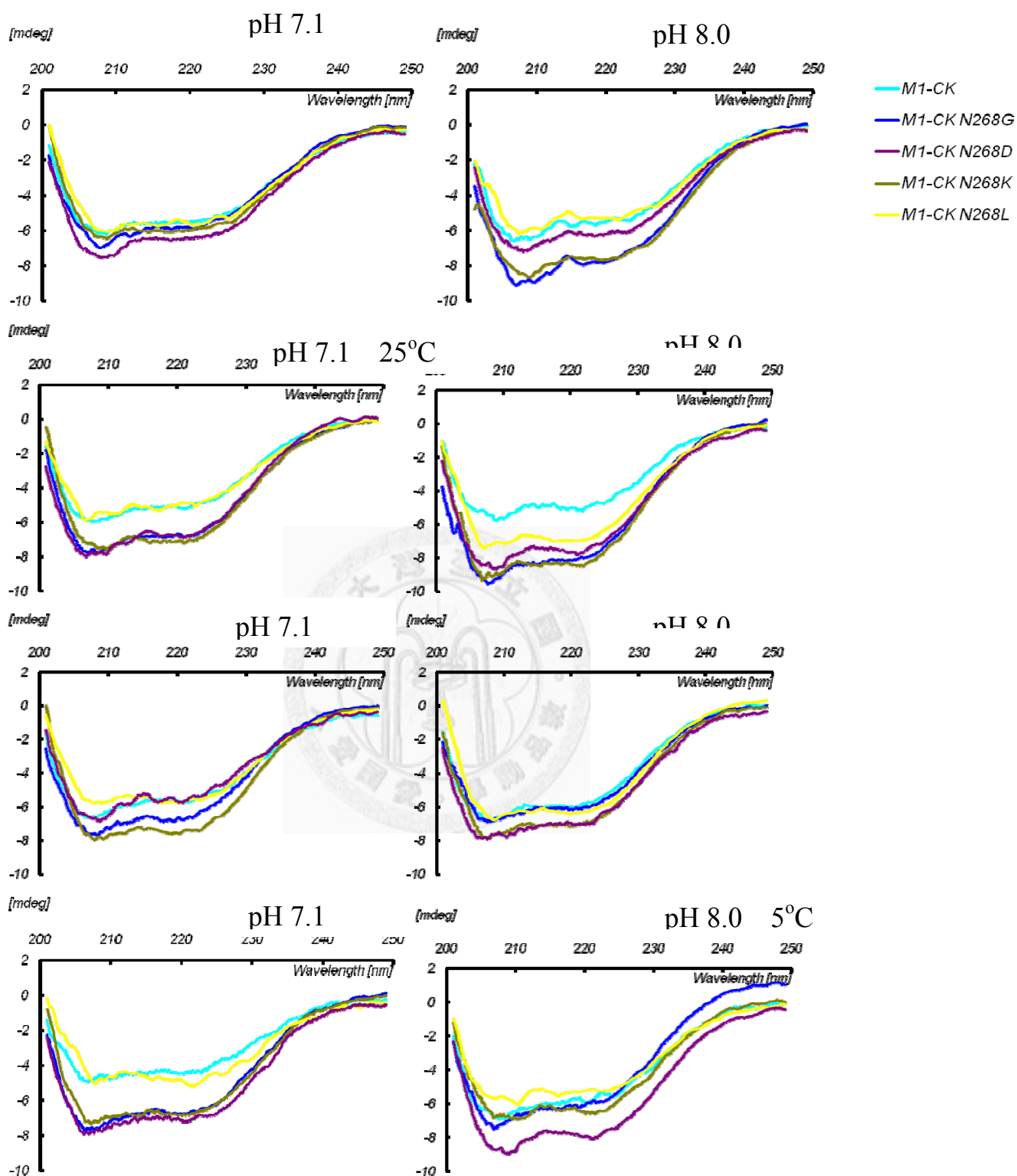


Figure 12. Spectra of far-UV circular dichroism (CD) spectroscopy of M1-CK (cyan), M1-CK N268G (blue), M1-CK N268D (purple), M1-CK N268K (green) and M1-CK N268L (yellow) at different temperature at pH 7.1 and 8.0 .

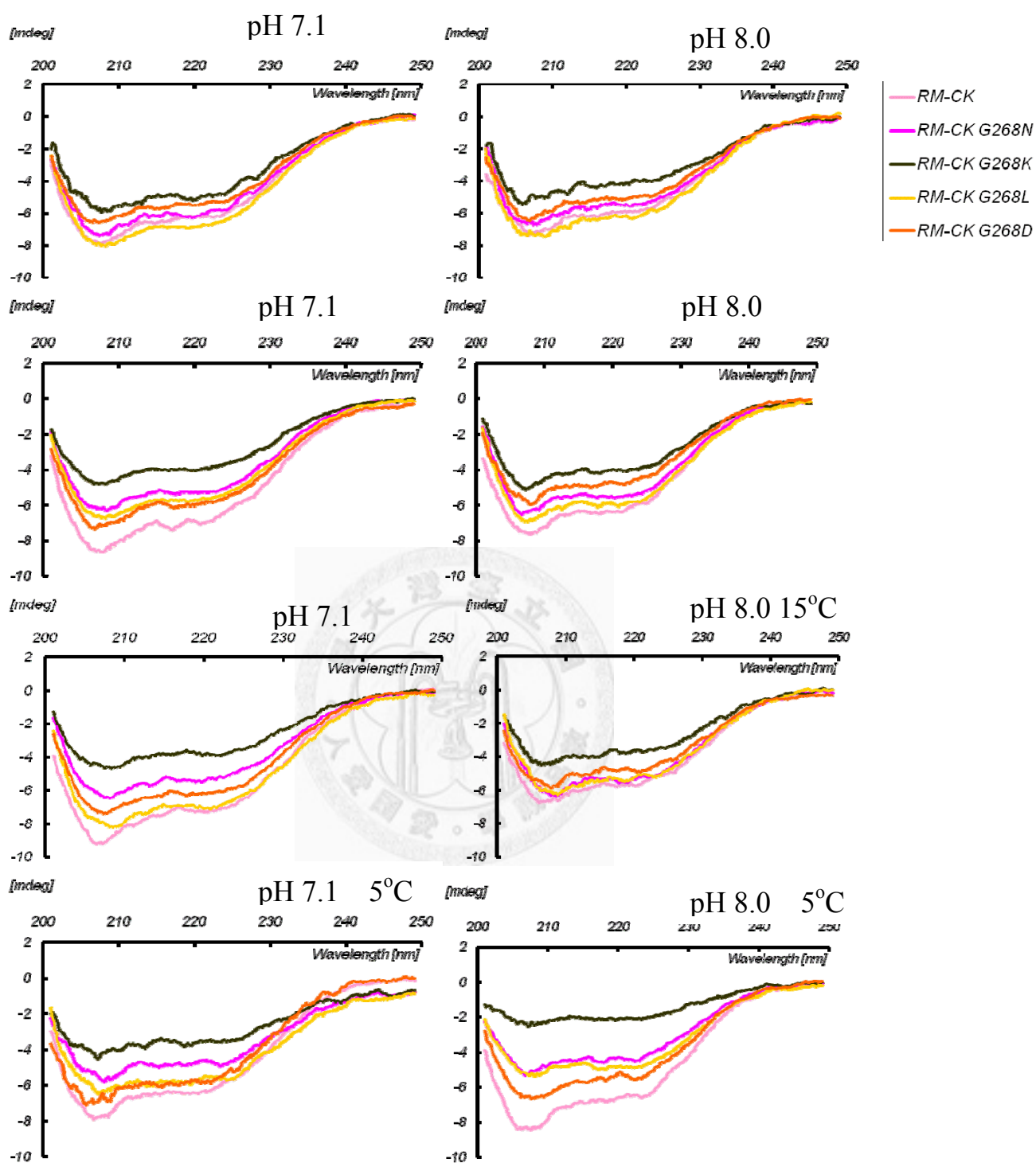


Figure 13. spectra of far-UV circular dichroism (CD) spectroscopy of RM-CK (pink), RM-CK G268N (magenta), RM-CK G268D (orange), RM-CK G268K (dark green) and RM-CK G268L (yellow) at different temperature at pH 7.1 and 8.0 .

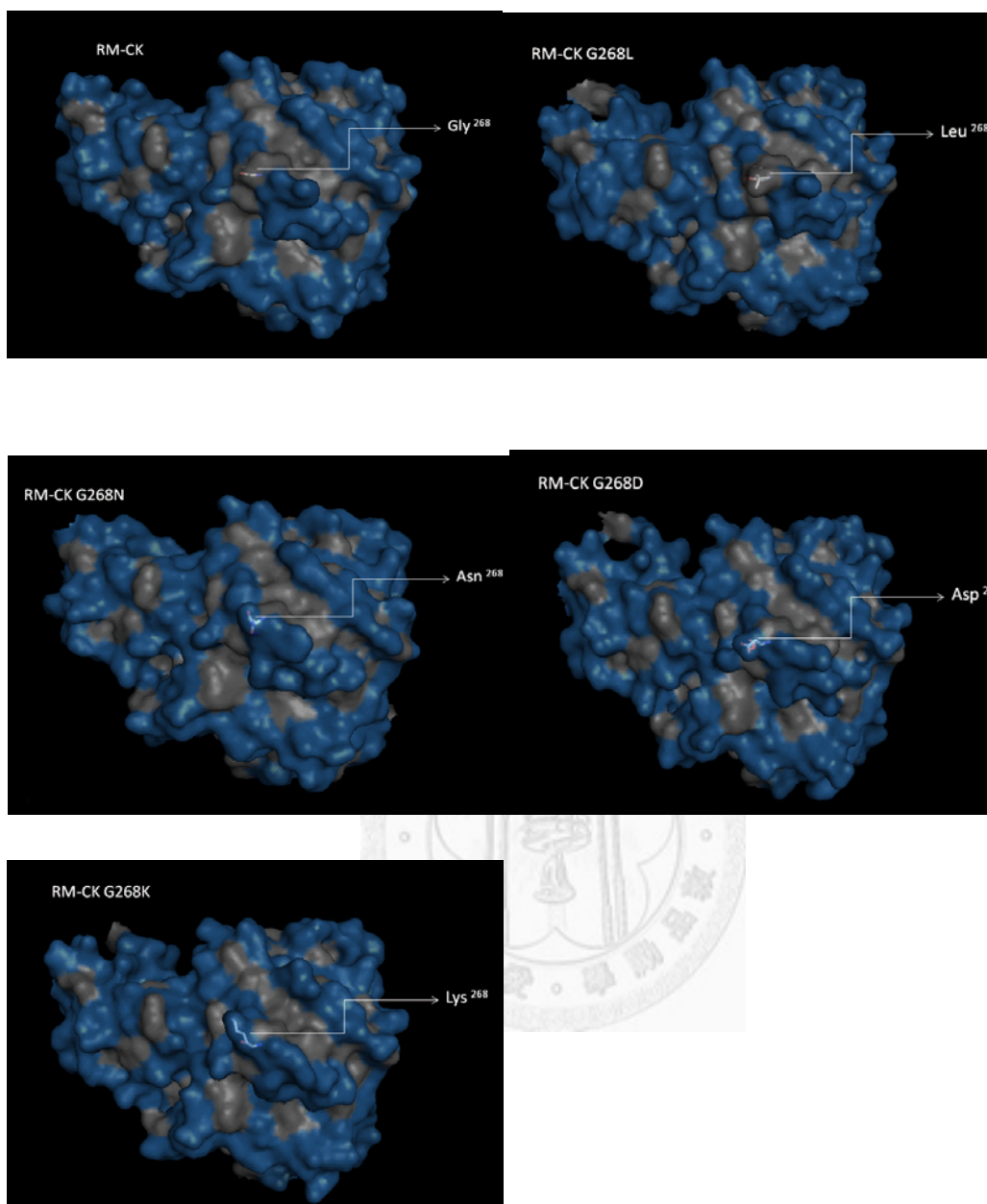


Figure 14. The hydrophilic and hydrophobic surface around residue 268 of RM-CK and its mutants. Hydrophilic surface was colored by blue, and hydrophobic surface was colored by grey.

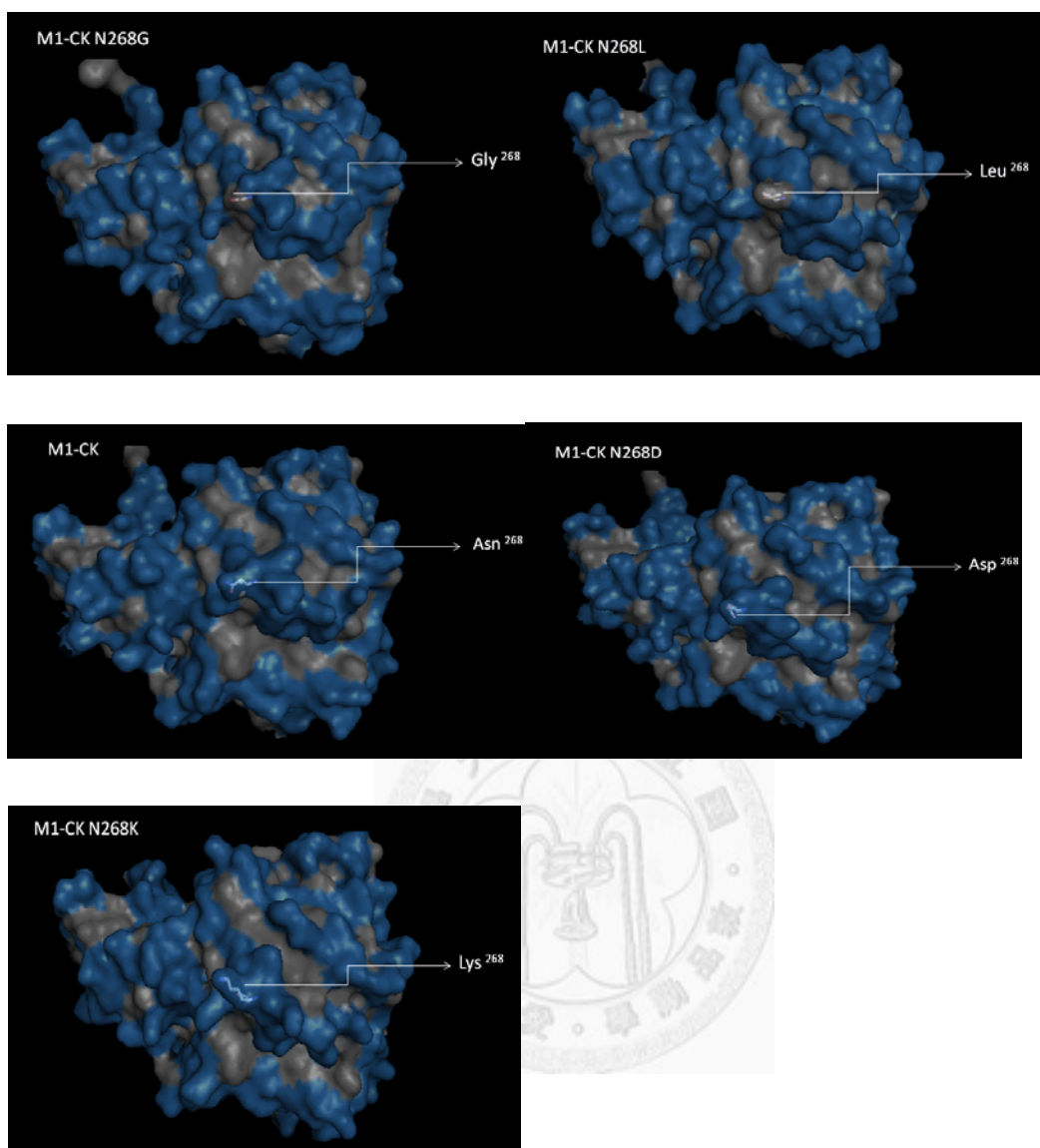


Figure 15. The hydrophilic and hydrophobic surface around residue 268 of M1-CK and its mutants. Hydrophilic surface was colored by blue, and hydrophobic surface was colored by grey.