The Generation of the Oxidized Form of Creatine Kinase Is a Negative Regulation on Muscle Creatine Kinase*

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Muscle creatine kinase (CK) is a crucial enzyme in energy metabolism, and it exists in two forms, the reduced form (R-CK) and the oxidized form (O-CK). In contrast with R-CK, O-CK contained an intrachain disulfide bond in each subunit. Here we explored the properties of O-CK and its regulatory role on muscle CK. The intrachain disulfide bond in O-CK was demonstrated to be formed between Cys74 and Cys146 by site-directed mutagenesis. Biophysical analysis indicated that O-CK showed decreased catalytic activity and that it might be structurally unstable. Further assays through guanidine hydrochloride denaturation and proteolysis by trypsin and protease K revealed that the tertiary structure of O-CK was more easily disturbed than that of R-CK. Surprisingly, O-CK, unlike R-CK, cannot interact with the M-line protein myomesin through biosensor assay, indicating that O-CK might have no role in muscle contraction. Through in vitro ubiquitination assay, CK was demonstrated to be a specific substrate of muscle ring finger protein 1 (MURF-1). O-CK can be rapidly ubiquitinated by MURF-1, while R-CK can hardly be ubiquitinated, implying that CK might be degraded by the ATP-ubiquitin-proteasome pathway through the generation of O-CK. The results above were further confirmed by molecular modeling of the structure of O-CK. Therefore, it can be concluded that the generation of O-CK was a negative regulation of R-CK and that O-CK might play essential roles in the molecular turnover of MM-CK.

In the living cells, continuous turnover of the structural, catalytic, and regulatory proteins are required to maintain the normal function. Two pathways are responsible for degradation of most of the cellular proteins, the lysosomal proteases and the ATP-ubiquitin-proteasome system (1, 2). The ubiquitin system is the main proteolytic system involved in intracellular catabolism of most abnormal, short-lived and long-lived muscle proteins (3). Studies in various cases of atrophy indicated that the activation of this pathway is primarily responsible for the rapid loss of muscle proteins (3, 4), which was further confirmed by that the two muscle specific E3ligases, MURF-1 and MURF-2, had been suggested to target a lot of myofibrillar proteins and energy metabolism enzymes for ubiquitin-dependent degradation (5). For the myofibrillar proteins, the dissociation from the myofibrillar complexes was the rate-limiting step in the degradation (3), but little is known about how the cytoplasmic proteins, including the enzymes required for ATP/energy production, in the muscle cells were recognized and degraded by the ubiquitin system.

Among the energy metabolism enzymes in the muscle cells, creatine kinase (EC 2.7.3.2) plays a significant role in energy homeostasis (6, 7). It catalyzes the reversible conversion from MgATP and creatine to MgADP and phosphocreatine, high energy phosphate able to supply ATP on demand. Muscle type CK (MM-CK) has the unique property to bind with the M-line of sarcomere mediated by the NH2-terminal lysine charge clamps (8). The catalytic activity of MM-CK is under elaborate regulation to meet its function in the muscle cells (9, 10). In the activation state of the muscle, the acidification in the microenvironment makes MM-CK bind with M-line protein (11) and function as an ATP supplier coupled with the myofibrillar actin-activated Mg2+-ATPase (12, 13). In the resting state of the muscle, MM-CK dissociates from the myofibril and catalyzes the formation of phosphocreatine to reserve energy (11). However, it is still unclear about how MM-CK is regulated at protein level and how MM-CK is degraded in vivo.

Here in the present study, we explored the regulation of MM-CK through the oxidized form of CK (O-CK), a form of CK first identified in 1994 (14). Different from the reduced form of CK (R-CK), O-CK contained an intrachain disulfide bond in each subunit, and it was about 15% of native MM-CK (14, 15). Through biochemical and functional analysis of O-CK, we found that the generation of O-CK was a negative regulation on MM-CK and that the degradation of MM-CK by ubiquitin-dependent pathway was fulfilled through the degradation of O-CK. The findings here disclosed a novel pathway for protein degradation by ATP-ubiquitin-proteasome system in the muscle cells, and it might provide useful information for under-

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2 The abbreviations used are: E3, ubiquitin-protein isopeptide ligase; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; CK, creatine kinase; MM-CK, muscle type creatine kinase; WT-CK, rabbit muscle creatine kinase; R-CK, reduced form of creatine kinase; O-CK, oxidized form of creatine kinase; HMy7–8, domains 7–8 of human myomesin; ANS, 1-anilinonaphthalene-8-sulfonate; GdnHCl, guanidine hydrochloride; DTT, dithiothreitol; MURF, muscle ring finger protein; PDI, protein disulfide isomerase.
standing the degradation of the cytoplasmic energy metabolism enzymes.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis**—The gene of rabbit muscle CK (Wt-CK) has been cloned into pET21b expression vector (16). Site-directed mutagenesis against the four cysteine residues in Wt-CK was carried out using the following mutagenic primers (mismatches with the template are underlined): C74S (FC74S, 5’-CGCGGGCTTCGCGGCGGTG-3’; RC74S, 5’-GGGCGGGCGGGCGGTG-3’), C146S (FC146S, 5’-GCCCCGCCTCCTCCTCGGGTG-3’; RC146S, 5’-GTCGAGGAGAATGC-3’), C254S (FC254S, 5’-CGGCCGCTTCTCGGTTGGTGCGAGAAGAC-3’), C283S (FC283S, 5’-GTTGGACGGGGAGCCGGAAGAC-3’). The mutants were cloned into pET21b (Novagen) and pCMV5-myc (Clontech) for protein expression and cell transfection, respectively.

**Protein Expression and Purification**—Wt-CK and the mutants were expressed in *Escherichia coli* BL21 [DE3]-pLysS (Stratagene, Heidelberg, Germany) and purified as described (16). The preparation of O-CK was according to the (orthophenanthroline)2Cu(II) method, as described previously (14). R-CK was prepared by dissolving the freeze-dried WT-CK in the buffer containing 10 mM DTT. R-CK was confirmed to show only the 43-kDa band on non-reduced SDS-PAGE immediately after desalting. R-CK was prepared immediately before use.

**Physico-chemical Analysis**—Far-UV CD spectra were measured using a Jasco 725 spectrophotometer, using a cell with a path length of 0.1 cm. Fluorescence spectra were collected on an F-2500 spectrofluorometer using a 1-ml cuvette, with excitation at 285 nm and emission in the range of 300–400 nm. Since ANS is non-fluorescent in polar solvents and exhibits a blue shift and significant increase in quantum yield when binding to the hydrophobic region of the protein (22), it has been extensively used as an extrinsic fluorescence probe (23). 10-fold molar excess of ANS was incubated with the samples for 30 min in the dark, and fluorescence was measured using a 1-ml cuvette, with excitation at 380 nm and emission in the range of 400–600 nm. All resultant spectra were collected at 25 °C. The final concentrations of the proteins were 0.2 mg/ml.

**Enzyme Kinetics**—CK activity was measured according to the pH-colorimetry method (24). The reaction mixture contained 24 mM creatine, 4 mM ATP, 5 mM magnesium acetate, and 0.01% thymol blue, in 5 mM glycine-NaOH buffer (pH 9.0). To determine the *K_m*, ATP values of the proteins, the concentration of creatine was kept constant at 24 mM, while the concentrations of ATP were 0.5, 0.8, 1.0, 1.5, 2.0, and 2.5 mM, respectively. To determine the *K_m*, creatine values of the proteins, the concentration of ATP was 4 mM, while the concentrations of creatine were 6, 8, 10, 12, 16, and 20 mM, respectively. The data were fit to the Michaelis-Menten equation to obtain the kinetic constants. Each kinetic constant was the average of at least three results.

**GdnHCl Denaturation**—All denaturation was carried out in 10 mM Tris-HCl (pH 8.0) at 25 °C overnight. Samples were prepared containing 0.2 mg/ml CK and a varied amount of GdnHCl ranging from 0 to 4 M. Then CD, intrinsic fluorescence, and ANS fluorescence were conducted to reflect the structural changes with the increase of GdnHCl concentration.

**Proteolytic Susceptibility of R-CK and O-CK by Trypsin and Protease K**—To detect the degradation rate of R-CK and O-CK by other proteases, the site-specific protease, trypsin, and the unspecific protease K were chosen. The final concentrations of CKs, trypsin, and protease K were 0.3, 1.0, and 0.2 mg/ml, respectively. Proteolysis by trypsin was conducted at 37 °C and by protease K at 25 °C. At certain intervals, 100 µl of the sample was loaded on a Superdex 200 10/300 GL column (fast protein liquid chromatography, GE Healthcare) for size exclusion chromatography analysis.

**Biosensor Assay**—Purified hMy7–8 was coupled to carboxymethylxetranate-coated biosensor chips (CM5, Biacore, Upsala, Sweden) following the manufacturer’s instructions. Briefly, carboxymethylxetranate curvettes were activated by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride/N-hydroxysuccinimide (EDC/NHS) and hMy7–8 was coupled at 0.1 mg/ml in 20 mM acetate buffer (pH 5.0). The protein density was 2000 response units. hMy7–8 interactions were measured in binding buffer (80 mM sodium propionate (pH 6.0), 10 mM imidazole, and 150 mM sodium chloride). Regeneration of the sensor curvette was achieved with 40 µl of 50 mM NaOH, followed by an 8-min washing with binding buffer.

**In Vitro Ubiquitination Reaction**—In vitro ubiquitination was performed as described previously (25) with some revisions. In brief, 1 µM Wt-CK or O-CK, 0.1 µM human E1, 1.6 µM UBCH5C, 2 µM MURF-PDI or PDI, and 1.2 mg/ml ubiquitin were incubated in 20 mM Tris-HCl (pH 7.6), 10 mM magnesium chloride, 100 mM potassium chloride, 5 mM ATP, and 5% glycerol with or without 10 mM DTT at 30 °C. At suitable intervals, samples were analyzed by SDS-PAGE and immunoblotting.

**RESULTS**

**The Intrachain Disulfide Bond of O-CK Was Formed between Cys74 and Cys146**—As is known, each subunit of O-CK has an intrachain disulfide bond (14, 15), but it is controversial about
which Cys residues are involved in the formation of the intra-chain disulfide bond. Wang et al. (14) deduced that Cys283, a residue crucial for activity, was not involved because O-CK still had considerable activity, while Hurne et al. (15) suggested the intrachain disulfide bond might be formed between Cys74 and Cys283. To clarify this issue, four site-directed mutants, C74S, C146S, C254S, and C283S, were constructed and analyzed on reduced and non-reduced SDS-PAGE. As was clearly shown in Fig. 1A, Wt-CK, C254S, and C283S contained R-CK and O-CK, but C74S and C146S consisted of only R-CK (Fig. 1A). Therefore it can be concluded that the intrachain disulfide bond was formed between Cys74 and Cys146.

To further explore whether the intrachain disulfide bond in O-CK was also formed between these two residues in the cells, the mutants were constructed into pCMV5-myc and transiently transfected into HEK 293T cells. The cells were harvested in non-reduced or reduced SDS loading buffer and analyzed by immunoblotting. Green fluorescent protein was also transfected as an internal control. The results (Fig. 1B) here further confirmed that the intrachain disulfide bond was indeed formed between Cys74 and Cys146.

The Generation of the Intrachain Disulfide Bond Decreased the Activity of CK—A kinetic analysis of the forward reaction (phosphocreatine formation) at 25 °C was performed to determine the kinetic constants of the CKs. The data were fit to the Michaelis-Menten equation to calculate the kinetic constants, as shown in Table 1. There was almost no difference between R-CK and O-CK in the \( K_m \) values for both ATP and creatine, suggesting the generation of O-CK does not affect the ability of CK to bind with the substrates. However, O-CK showed a decrease in its catalytic ability, and its specific activity was only 76% of that of R-CK.

The Structure of O-CK Was More Easily Disturbed by GdnHCl Denaturation—The physico-chemical properties of R-CK and O-CK were explored by CD (Fig. 2A), intrinsic fluorescence (Fig. 2B) and ANS fluorescence (Fig. 2C). The results here showed that O-CK, compared with R-CK, showed decreased secondary structures (Fig. 2A), a red shift in intrinsic fluorescence emission wavelength maximum (\( E_{max} \)) (Fig. 2B) and increased hydrophobic surface exposure. As a mixture of R-CK (85%) and O-CK (15%), Wt-CK showed the properties between R-CK and O-CK. Therefore the results indicated that O-CK might be structurally unstable.

To further explore the structural stabilities of R-CK and O-CK, the proteins were subjected to GdnHCl denaturation, as monitored by CD (Fig. 3A), intrinsic fluorescence (Fig. 3B), and ANS fluorescence (Fig. 3C). As shown in Fig. 3, the greatest differences between R-CK and O-CK were in the region from 0 to 1 M GdnHCl, during which both R-CK and O-CK had steep burst in the spectra as was shown in the insets. The steep transition has been attributed to dimer dissociation (26). As indicated by changes in secondary structures, tertiary structures, and hydrophobic surface exposure, the steep transition in the structures of O-CK occurred at lower concentrations of GdnHCl than that of R-CK. Then the biophysical results above indicated that the stability of O-CK against GdnHCl denaturation was weaker than that of R-CK, and the results herein suggested that the formation of the intrachain disulfide bond

![FIGURE 1. Identification of the intrachain disulfide bond of O-CK. A, non-reduced and reduced SDS-PAGE analysis of the purified Wt-CK, O-CK, and the mutants. B, Western blot analysis of Wt-CK and the mutants transiently expressed in HEK 293T cells. Green fluorescent protein (GFP) was used as an internal control.](image)

![FIGURE 2. Far-UV CD (A), intrinsic fluorescence (B), and ANS fluorescence (C) analysis of Wt-CK, R-CK, and O-CK. Lines 1–3 are Wt-CK, R-CK, and O-CK, respectively. The final concentrations of the proteins were 0.2 mg/ml.](image)

![TABLE 1 Kinetic analysis of the enzymes](table)
had a slight adjustment of the domain-domain and dimer interactions, which gave rise to a relatively unstable form of CK.

O-CK Was More Susceptible to Proteolysis by Both Trypsin and Protease K—To further testify whether the generation of the intrachain disulfide bond in O-CK led to structural instability, proteolysis of Wt-CK and O-CK by trypsin and protease K was carried out. The time courses of proteolysis of Wt-CK and O-CK by trypsin and protease K were shown in Fig. 4, A and B, respectively. As was clearly shown in the figures, dimeric CK gradually decreased with increasing time and, correspondingly, four new peaks occurred and increased with time with the molecular mass of about 37, 24, 18, and 6 kDa. The proteolysis pattern of O-CK was similar to that of Wt-CK (Fig. 4B), but the decrease of dimeric CK or increase of the new peaks obviously sped up. According to the decrease of the dimeric peak (Fig. 4C), the percentage of dimeric Wt-CK, at any given time, was higher than that of O-CK. After 8 h, the intact Wt-CK was 58.8%, while the remained O-CK was only 38.3%.

The proteolytic pattern of Wt-CK by protease K was quite different from that by trypsin (Fig. 4D). The dimeric Wt-CK gradually decreased with increasing time, and after about 2 h, a 74-kDa peak appeared. Then the 74-kDa peak gradually increased with the decrease of the 86-kDa peak, and after 4 h, the 74-kDa peak completely replaced the 86-kDa peak. The behavior of protease K observed here was somewhat unspecific, but it also showed some site-specificity, as has been
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FIGURE 5. Biosensor analysis of the interactions of Wt-CK and O-CK with myomesin. The central domain of human myomesin, hMy7–8, was immobilized on the chip. The binding curves of Wt-CK were shown in solid lines, and the concentrations were as designated. The dashed line was the curve obtained with O-CK at 11.6 μM. RU, relative units.

FIGURE 6. In vitro ubiquitination analysis was performed to demonstrate that MURF-1 targeted CK for ubiquitination. Ubiquitinated CK was detected with anti-CK (upper) and anti-ubiquitin (lower) antibodies. The molecular marker was as indicated.

FIGURE 7. In vitro ubiquitination analysis of Wt-CK and O-CK by MURF-1. In the upper panels, the reaction buffer did not contain DTT. In the lower panels, Wt-CK and O-CK were preincubated with 50 mM DTT, and the final concentration in the buffer was 10 mM.

described in a previous study (27). The proteolysis of O-CK by protease K was much quicker than that of Wt-CK, and dimeric O-CK completely disappeared after 2.5 h of proteolysis (Fig. 4E). As was illustrated in Fig. 4F, the residual intact Wt-CK was more than that of O-CK at any time after proteolysis by protease K.

O-CK, Unlike R-CK, Cannot Interact with the M-line Protein Myomesin—It has long been demonstrated that a significant amount of cytosolic MM-CK is specifically bound to the myofibrillar M-line (28–30). Recently, it was found that MM-CK can interact with the central domains of the M-line proteins myomesin and the closely related M-protein (11). Domains 7–8 of myomesin play a crucial role in the interaction with CK, and it has also been demonstrated that this interaction is not strictly species-specific (8, 11, 31). Since there is no published cDNA sequence of rabbit muscle myomesin, human myomesin fragment (hMy7–8) was cloned, expressed and purified to test the binding affinity of R-CK and O-CK with M-line protein. Consistent with the previous report (11), Wt-CK bound with hMy7–8 in a concentration-dependent manner as monitored by biosensor chips (Fig. 5). However, O-CK could not bind with hMy7–8 even at a concentration as high as 11.6 μM (Fig. 5). Since the M-line bound MM-CK functions as an ATP regenerator when the muscle is in rapid metabolic activity, the inability of O-CK to bind with M-line protein might be an implication that O-CK might have no genuine physiological roles, at least in muscle contraction.

O-CK Is More Sensitive to the ATP-Ubiquitin-Proteasome Degradation Pathway—According to a previous study by yeast two-hybrid assay, CK interacted with MURF-1, an E3 ligase of the ATP-ubiquitin-proteasome system (5), which indicated that CK might be degraded through this special system. Here, we purified human E1, E2 (UBCH5C), and E3 (MURF-1), tested the hypothesis above, and compared the degradation rate of R-CK and O-CK by ATP-ubiquitin-proteasome system. As was shown in Fig. 6, both Wt-CK and O-CK can be ubiquitinated when the reaction buffer contained E1, UBCH5C, MURF-1, and ubiquitin, but elimination of anyone of them failed to produce ubiquitinated CK. Therefore, the results here strongly demonstrated that CK was a specific substrate of MURF-1 and that CK might be degraded through the ATP-ubiquitin-proteasome system.

Since CK was a substrate of the ATP-ubiquitin-proteasome system, experiments were carried out to explore which form of CK was ubiquitinated at a faster rate. To ensure that the reactions were carried out under the same conditions (without DTT), Wt-CK was used to be a reflection of R-CK, since 85% of Wt-CK is R-CK. As was shown in the upper panel of Fig. 7, Wt-CK showed a slow decrease with increasing time, while O-CK decreased at a much faster rate.

To test the ubiquitination rate of R-CK and to explore whether reduction of O-CK to R-CK can rescue the quick ubiquitination of O-CK, both Wt-CK and O-CK were preincubated with 50 mM DTT for 30 min to destroy the intrachain disulfide bonds in O-CK, followed by the ubiquitination assay in the reaction buffer containing 10 mM DTT. Surprisingly, under
such conditions, CK showed almost no decrease with increasing time, which strongly suggested that R-CK might be ubiquitinated at a fairly slow rate. Therefore, O-CK was much more sensitive to ubiquination than R-CK, and O-CK might play some essential roles in the turnover of muscle CK. 

**Molecular Modeling the Structure of O-CK**—Since the intrachain disulfide bond of O-CK is formed between Cys74 and Cys146 (Fig. 1), we made a molecular modeling of the structure of O-CK based upon the published crystal structure of R-CK (Fig. 8A) (32). O-CK, like R-CK, also existed as a dimer, consistent with the results from fast protein liquid chromatography (Fig. 4). As a whole, the molecular profile of O-CK did not have large changes with R-CK, but dramatic changes occurred in some special regions according to structural alignment of R-CK and O-CK (Fig. 8, B and C). The most obvious change in O-CK is in the dimerization interface (Fig. 8B). In O-CK, Asp54 of Subunit I and Arg148 of Subunit II did not form the hydrogen bonds as those in R-CK (Fig. 8B) (32–34). Therefore, this might be a major reason for why O-CK was structurally unstable as explored by GdnHCl denaturation (Fig. 3) and proteolysis (Fig. 4).

Another interesting finding is the movement in the orientations of Lys25 and Lys116, as highlighted in Fig. 8C. The isozyme-specific interaction of MM-CK with M-line protein myomesin and M-protein had been demonstrated to be mediated by four NH2-terminal lysine residues that act pair-wise as weak (Lys9/Lys25) and strong (Lys105/Lys116) binding sites (8, 11). Although both R-CK and O-CK contained these four residues, their binding abilities with M-line proteins were completely different. Therefore, the conformational states of these four Lys residues might be essential for the isozyme-specific interaction. Compared with those in R-CK (Fig. 8C), Lys9 and Lys105 in O-CK did not change much in the spatial orientations, but the side chains of Lys25 and Lys116 in O-CK changed greatly in their orientations, which might be the key factor for O-CK to lose the ability to bind with myomesin (Fig. 5).

It is also noteworthy that the orientation of Cys283 in O-CK has moved compared with that in R-CK (Fig. 8C), and this might be a cause for the decrease in its catalytic activity. In addition, the residues from Ser199 to Ser205 of O-CK do not form α-helices as those in R-CK, which might be a good explanation for the decrease in the secondary structures in O-CK.

**DISCUSSION**

The past decades have witnessed numerous studies on the degradation of muscular proteins, but the efforts were mainly focused on the myofibrillar proteins. In a proposed model for turnover of skeletal muscle proteins, degradation rates are determined by the rate of dissociation of protein subunits from the myofibrillar matrix (35). The dissociation of myosin, actin, and tropomyosin from the myofibrils has been demonstrated to be the rate-limiting step for their degradation (3). MM-CK has also been demonstrated to be able to associate with and dissociate from M-line, which has been shown to be strongly pH-dependent (11), but it is unlikely that MM-CK is degraded as those myofibrillar proteins, considering its special role in the muscle cells. Different from the structural proteins in myofibrils, CK has to function in an association/dissociation manner to meet with the energy state of the muscle cells. The role of the dissociated MM-CK is to catalyze the formation of phosphocreatine
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to reserve energy for the cell. Therefore, it is not reasonable that the dissociated MM-CK is degraded immediately. Then there must be some other ways for the degradation of MM-CK in the muscle cells.

Interestingly the findings here indicated that MM-CK might be degraded through the generation of O-CK, a form of CK which contained a disulfide bond in each subunit. O-CK showed a decrease in catalytic activity and structural stability. What is even surprising is that the intrachain disulfide bonds made O-CK unable to bind with M-line (Fig. 5), indicating that O-CK might have no physiological role in muscle contraction. Furthermore, O-CK can be rapidly ubiquitinated by MURF-1, but R-CK can hardly be ubiquitinated (Fig. 7). All these findings indicated that O-CK might be a negative regulation on muscle CK and that the degradation of MM-CK by the ATP-ubiquitin-proteasome might be through the conversion from R-CK to O-CK. Possibly, certain ubiquitin-conjugating enzymes, like MURF-1, would recognize the conformation of O-CK as an “abnormal” one. Once generated, O-CK would be rapidly ubiquitinated and degraded by the proteasome. Therefore the generation of O-CK must play essential roles in the protein degradation of MM-CK.

Different from the common rule that the disulfide bonds contributed to stabilize the proteins, the intrachain disulfide bonds in O-CK destabilized this special form of CK (Figs. 3 and 4). Then what are the possible reasons? For one thing, the large scale movement of Cys74 and Cys146 led to dramatic changes in some special regions, especially the dimerization interface (Fig. 8), and it thus affected the structural stability of O-CK. For another, the formation of the intrachain disulfide bonds changes the orientations of some special amino acids, like Cys283 and the Lys clamps for effective binding with M-line, and therefore it led to the decrease in catalytic activity and loss of function in muscle contraction. In addition, the formation of the intrachain disulfide bond between Cys74 and Cys146 destroyed the microenvironment of these two residues and thus destabilized the structure of O-CK. Our recent work showed that Cys74 and Cys146 played essential roles in maintaining the structure of MM-CK and that small changes in these residues can lead to the decrease in structural stability (36, 37).

According to the crystal structure of rabbit MM-CK (32), Cys74, Cys146, and Cys283 are near in their oriental positions. Then why Cys74 and Cys146, with a distance of 18 Å, were selected to form the intrachain disulfide bond instead of Cys74 and Cys283 (7 Å) or Cys146 and Cys283 (16 Å)? It is most probable that the intrachain disulfide bond is a result of the intramolecular movement, since Cys74 and Cys146 are both in the flexible regions. Besides, CK itself is a very flexible molecule, which is well demonstrated by the fact that CK showed a large movement in two loops upon binding with its substrates (38). Then it is reasonable for Cys74 and Cys146 to form the intrachain disulfide bond.

To have an overall view of the turnover of MM-CK and the possible role of O-CK, we proposed a working model (Fig. 9). In the muscle cells, the mRNA of CK is translated into R-CK in the cytosol after a folding process. When the muscle cells are in activation, the ATPase hydrolyzes a large amount of ATP and acidifies the microenvironment. The lower pH in the cytosol promotes R-CK to bind with M-line protein, and the bound R-CK catalyzes the hydrolysis of phosphocreatine to generate ATP based on the demands of myofibrillar actin-activated Mg2+-ATPase. When the muscle cells are at rest, the pH in the microenvironment will rise to greater than 7.0, and then R-CK will dissociate from the M-line protein. R-CK in such a state fulfills its role to reserve energy by producing the high energy phosphate, phosphocreatine. Under both conditions, R-CK can be converted into O-CK through some unknown mechanism, maybe oxidation. Once O-CK is generated, it is recognized and degraded rapidly by the ATP-ubiquitin-proteasome system, and this is the major pathway of the degradation of MM-CK. In such a way, MM-CK finishes its life cycle from the newly synthesized polypeptides, to the active R-CK, then to O-CK, and finally to be degraded into free amino acids. In such a process, the generation of O-CK is an important part in the turnover of MM-CK.

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