# Mutation of conserved active-site threonine residues in creatine kinase affects autophosphorylation and enzyme kinetics

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Muscle-type creatine kinase (MM-CK) is a member of an isoenzyme family with key functions in cellular energetics. It has become a matter of debate whether the enzyme is autophosphorylated, as reported earlier [Hemmer, Furter-Graves, Frank, Wallimann and Furter (1995) Biochim. Biophys. Acta **1251**, 81–90], or exclusively nucleotidylated. In the present paper, we demonstrate unambiguously that CK is indeed autophosphorylated. However, this autophosphorylation is not solely responsible for the observed microheterogeneity of MM-CK on two-dimensional isoelectric focusing gels. Using phosphoamino-acid analysis of $^{32}$P-labelled CK isoforms, phosphothreonine (P-Thr) residues were identified as the only product of autophosphorylation for all CK isoenzymes. The phosphorylated residues in chicken MM-CK were allocated to a region in the vicinity of the active site, where five putative phosphorylation sites were identified. Site-directed threonine–valine-replacement mutants reveal that autophosphorylation is not specific for one particular residue but occurs at all examined threonine residues. The enzyme kinetic parameters indicate that the autophosphorylation of CK exerts a modulatory effect on substrate binding and the equilibrium constant, rather than on the catalytic mechanism itself.

Key words: active site, autophosphorylation, creatine kinase, threonine.

## INTRODUCTION

Creatine kinase (CK; EC 2.7.3.2) is an enzyme that catalyses the reversible transfer of the high-energy phosphoryl group of phosphocreatine to MgADP to regenerate MgATP [1,2]. The enzyme exists in different isoforms, which show tissue-specific expression and are localized subcellularly in an isoenzyme-specific manner [3,4]. Two isoforms are restricted to the mitochondrial intermembrane space, whereas the other two isoforms are abundant cytosolic proteins [5,6]. All cytosolic isoenzymes are dimers, whereas the mitochondrial isoforms form mainly octamers. The 'muscle-type' isoenzyme MM-CK is predominantly found in differentiated skeletal muscle [7,8], whereas the 'brain-type' BB-CK is found in brain, smooth muscle and heart [9,10].

When analysed by isoelectric focusing (IEF) on two-dimensional gels, the cytosolic isoforms of chicken and other vertebrate species can be separated into a number of subspecies, with slightly different isoelectric points but identical apparent molecular mass [11,12]. This microheterogeneity of MM-CK and BB-CK is not specific for certain tissues or developmental stages. Instead it was found as a constant pattern of CK expression [11,12].

Previously, we have shown [13] that cytosolic CK isoforms of chicken and rabbit share the common characteristics to autophosphorylate *in vitro*. This phosphorylation was demonstrated to be an intramolecular mechanism, showing first-order kinetics with respect to the enzyme concentration. Autophosphorylation depends on bivalent cations, i.e. $Mg^{2+}$ or $Mn^{2+}$, as well as on the native enzyme conformation but not on substrate turnover and therefore does not represent a phospho-intermediate of the enzymic reaction [14]. Recently, autophosphorylation of CK has been challenged, claiming that CK is instead only nucleotidylated within the active site but not autophosphorylated [15]. However, in this study, we demonstrate unambiguously that CK is indeed autophosphorylated at defined threonine residues in the vicinity of the active site. The kinetic data of site-specific threonine-replacement mutants suggest a more modulatory role of these threonines on substrate binding rather than a direct effect on enzyme catalysis itself.

## MATERIAL AND METHODS

### *Escherichia coli* strains, plasmids and DNA manipulation

*E. coli* strain BL21 (DE3) pLysS and expression vector pET3b were used as described earlier for M- and B-CK [3]. pRF5 is identical with pET3b except for a deleted *Eco*RV–*Eco*RI fragment. *E. coli* XL1 blue, media and standard DNA manipulations were used as described previously [16]. The construction of plasmid pT17 containing the chicken M-CK cDNA [17,18] and pT23 containing the chicken $B_b$-CK cDNA [19] has also been described previously [20].

Polymerase-chain reaction was used for site-directed mutagenesis of chicken M-CK. The site-specific mutations were introduced by using the inverse PCR method [21] and appropriate oligonucleotides that were 5′-phosphorylated. Site-directed mutagenesis was performed using Pfu DNA polymerase (Stratagene). The purified PCR products were self-ligated using T4 DNA ligase (FPLCpure, Pharmacia Biotech) and transformed into *E. coli* XL1 blue. All CK mutants were finally checked by DNA sequencing.

### Protein sources

Rabbit MM-CK was from Boehringer Mannheim, Germany. Chicken MM, BB and the mutated CKs were overexpressed in *E. coli* BL21 pLys according to a standard procedure and purified to homogeneity as described elsewhere [3].

Abbreviations used: CK, creatine kinase; DTT, dithiothreitol; IEF, isoelectric focusing; MM-CK, muscle-type CK dimer; BB-CK, brain-type CK dimer; M-CK, muscle-type CK subunit; B-CK, brain-type CK subunit; PCr, phosphocreatine.

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### Autophosphorylation assay

For autophosphorylation, the procedure used was an adaptation of a previously described method [13]. Routinely, 250 $\mu$g of wild-type CK or mutant protein were incubated in AP-buffer (100 mM glycylglycine, 2 mM magnesium acetate, 0.5 mM EDTA, 2 mM dithiothreitol (DTT), pH 8.4) at a concentration of about 1 mg/ml for 20 min at 37 °C. Autophosphorylation was started by adding a substoichiometric amount (molar ratio 1:175 of ATP/protein) of 50 $\mu$Ci [$\gamma$-$^{32}$P]ATP (10 mCi/ml) and incubation was continued for another 2.5–5 h at 30 °C. Unreacted [$\gamma$-$^{32}$P]ATP was removed by size-exclusion chromatography on Sephadex G-25 columns, equilibrated in 50 mM ammonium bicarbonate, pH 7.9, for lyophilization. For intermittent storage, lyophilized samples were kept at 4 °C. Special care was taken to provide strictly the same reaction conditions and the same handling times with all samples of one experimental series.

### Two-dimensional IEF gels

The two-dimensional electrophoresis method used was an adaptation of previously described methods [22–26] and were optimized to suit our experimental requirements [27].

In brief, the isoelectric separation in the presence of carrier ampholytes (first dimension) was performed in a model 175 tube cell (Bio-Rad, Glattbrugg, Switzerland) using a Power PAC 3000 or a model 3000/300 xi power supply (both from Bio-Rad). Glutamic acid (10 mM, pH 3.1) was used as the anode buffer and 10 mM ethylendiamine, pH 11.2, as the cathode buffer. The first-dimension gel solution (13.5 ml) was composed of 5 % acrylamide/bisacrylamide (30:0.8), 9 M urea (Schwarzmann, ultrapure), 2.2 % carrier ampholytes and 2.5 % detergent mix (30 % CHAPS, 15 % Nonidet P40). The carrier ampholytes used were a mixture consisting of ampholyte pH 5 → 8 (Pharmalyte®; Pharmacia, Uppsala, Sweden) and ampholyte pH 3 → 10 (Sevalyte® 'IsoDALT'; Serva, Heidelberg, Germany), each a 40 % solution, resulting in a optimal resolution for chicken MM-CK. After the urea was dissolved, the gel solution was degassed and filtered through a 0.2 $\mu$m filter unit. Polymerization was initiated by adding 30 $\mu$l 10 % ammonium persulphate solution and 10 $\mu$l *N*,*N*,*N*′,*N*′-tetramethylethylenediamine. Samples for the first dimension were composed of 31 $\mu$l protein sample, 7.5 $\mu$l 200 mM DTT and 15 $\mu$l detergent mix (30 % CHAPS, 15 % Nonidet P40). The amount of protein used depended on the subsequent detection method and the protein composition. Routinely, 25 $\mu$g protein was used from a tissue extract and 2.5 $\mu$g from a purified protein sample for detection by silver staining or immunodetection. For Coomassie Blue staining, 3–5-fold of the amount was used. In addition, comigration of two-dimensional SDS/PAGE standards (Bio-Rad) or carbamylated glyceraldehyd-3-phosphate dehydrogenase (Carbamylyte™ GAPDH; Pharmacia) was used to determine precisely the isoelectric point (pI). The molecular mass of these sample proteins served also to match two-dimensional gels.

### Quantification of radioactivity incorporated into CK proteins

To analyse radioactively labelled proteins, the corresponding material was separated by SDS/PAGE or two-dimensional IEF. The corresponding gel was either electroblotted on to Immobilon-P membrane (Millipore, Bedford, MA, U.S.A.) and visualized by standard Coomassie Blue R-250 staining (two-dimensional IEF) or directly dried in a gel dryer (SDS/PAGE). The radioactive protein pattern was then visualized with the PhosphorImager™ (Molecular Dynamics, Sunnyvale, CA, U.S.A.) and quantified using the MultiQuant™ software (Molecular Dynamics).

### CK activity measurements

Specific enzyme activities were determined in the pH-stat assay [28,29], measuring the initial velocity of the reverse reaction (ATP production) at pH 7.0 and 25 °C in the presence of 4 mM ADP, 10 mM phosphocreatine (PCr), 10 mM $MgCl_2$, 75 mM KCl, 0.1 mM EGTA and 1 mM $\beta$-mercaptoethanol. The Michaelis–Menten constants $V_{max,rev}$, $K_m^{ADP}$ and $K_m^{PCr}$ were determined by measuring the initial velocity of the reverse reaction under the same conditions as above, but varying the ADP concentration between 0.05 and 10 mM, and the PCr concentration between 0.25 and 50 mM, sometimes even up to 100 mM.

The specific enzyme activities in the forward reaction (PCr production) were measured under a $N_2$ atmosphere at pH 8.0 and 25 °C in the presence of 10 mM ATP, 45 mM creatine, 16 mM magnesium acetate, 75 mM KCl, 0.1 mM EGTA and 1 mM $\beta$-mercaptoethanol. The concentration of magnesium acetate was always in a 1 mM excess over the ATP concentration. The kinetic constants $V_{max}$ and $K_m$ were obtained by a least-squares fitting of the substrate-dependent reaction velocities to the Michaelis–Menten equation using the software KaleidaGraph™ V3.0.5 (Abelbeck Software).

### Cleavage by CNBr

Specific cleavage at methionine residues by CNBr was based on previously described methods [30]. MM-CK protein (200 $\mu$g) was autophosphorylated as described above. The lyophilized pellet was resolubilized in 0.8 ml 70 % formic acid and 1 % phenol. The protein sample was reacted with a 100-fold molar-excess CNBr (about 0.6 mg) over total methionine content in MM-CK for 36 h at 22 °C in the dark. The reaction was then quenched by lyophilization to remove the formic acid. Potentially developing CNBr vapour was detoxified by reacting it with ammonia, from which a solution was co-lyophilized with the sample. The digest was then resolubilized in 0.5 ml of distilled water and relyophilized, for washing. The cleavage products were then analysed by electrophoresis on a 16.5 % Tricine/SDS gel [31].

### Monitoring of the phosphoamino-acid stability

The stability of the $^{32}$P-label in autophosphorylated chicken MM-CK under acidic and alkaline conditions was monitored according to a slightly modified method described by Hemmer et al. [13]. Identical samples of $^{32}$P-autophosphorylated chicken MM-CK were separated by two-dimensional IEF and transferred electrophoretically to Immobilon-P (Millipore) PVDF membranes as described above. Individual Immobilon™ membranes were then transferred to different solutions exposing the blotted protein to various pH conditions and incubated at 55 °C for 2 h: 50 mM Tris, 100 mM NaCl (pH 8.0; control); 1.0 M KOH (pH 13; alkaline conditions); 0.15 M $HClO_4$ (pH 0.9; acidic conditions). The membranes were then neutralized by rinsing once for 1 min in 50 mM Tris/HCl, pH 8.0, 100 mM NaCl, once for 1 min in 200 mM Tris/HCl, pH 8.0 and twice in deionized water. The remaining radioactivity was determined as described above.

### Acid hydrolysis of CK and TLC for the identification of phosphoamino acids

CK protein (50 $\mu$g) was autophosphorylated as described above. Unreacted [$\gamma$-$^{32}$P]ATP was removed using a home-made Sephadex G-25 column, equilibrated in 50 mM ammonium bicarbonate, pH 7.9 and the purified protein lyophilized. The $^{32}$P-labelled protein was then resuspended in 250 $\mu$l of 50 mM ammonium bicarbonate, 0.5 % SDS, 5 mM DTT, supplemented with 25 $\mu$g proteinase K and 25 $\mu$g pronase and incubated at 37 °C for 2 h. A 6 M HCl solution was made by adding an equivolume of 12 M HCl and hydrolysed for 2 h at 110 °C in a screw-cap tube. The acid hydrolysate was then lyophilized and the residues washed twice by resolubilization in 100 $\mu$l distilled water and relyophilization. The hydrolysate was then resuspended in 50 $\mu$l distilled water and the samples were subjected to TLC using an ascending solvent system consisting of *i*-butyric acid/0.5 M $NH_4OH$ (5:3; v/v) according to a method described previously [32,33]. The separation was performed on cellulose thin-layer plates without fluorescence indicator (20 × 20 cm; 100 $\mu$m layer thickness; Merck, Dietikon, Switzerland). Amino acids and reference phosphoamino acids (Sigma, St Louis, MO, U.S.A.) were detected with ninhydrin spray (Sigma) and $^{32}$P-labelled phosphoamino acids were detected by autoradiography.

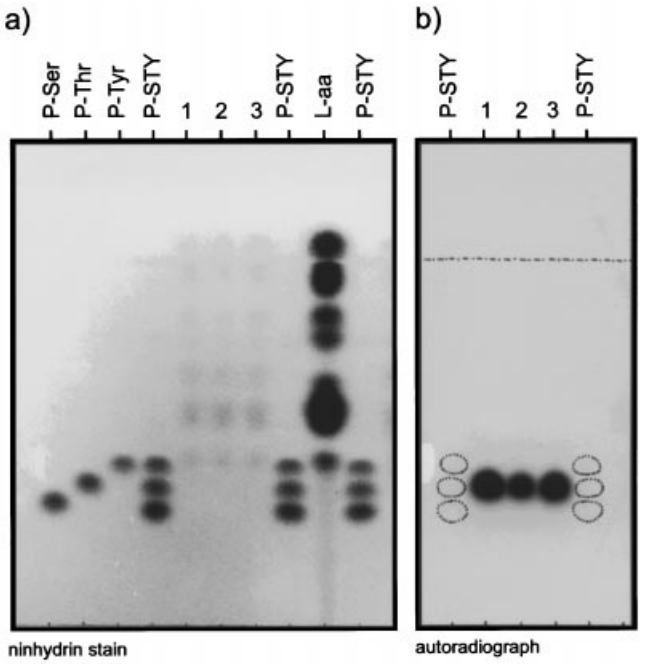


**Figure 2 Identification of phosphothreonine in autophosphorylated CK isoforms**

Separation of CK hydrolysates and reference phosphoamino acids on cellulose thin-layer plates in the ascending solvent system *i*-butyric acid/0.5 M $NH_4OH$ (5:3). References were visualized by ninhydrin staining and the $^{32}$P-labelled phosphoamino acid revealed by autoradiography. (**a**) Thin-layer plate of chicken CK isoenzyme hydrolysates and (**b**) the corresponding autoradiography. Lane 1, chicken Mib-CK; lane 2, chicken BB-CK; lane 3, chicken MM-CK. P-Ser, phosphoserine; P-Thr, phosphothreonine; P-Tyr, phosphotyrosine; P-STY, mixture of all three reference phosphoamino acids; L-aa, reference mixture of all 20 L-amino acids.

## RESULTS

### Autophosphorylation of MM-CK

It is a long-standing debate whether post-translational modification by phosphorylation is the major cause for the pronounced heterogeneity of cytosolic CK seen in IEF gels. To examine the extent of autophosphorylation in MM-CK the protein was incubated with [$\gamma$-$^{32}$P]ATP and separated by two-dimensional IEF (Figure 1).

The resulting spot pattern showed two major basic spots which were not radioactively labelled and four $^{32}$P-labelled subspecies. Interestingly, the two major unlabelled spots and the successive four labelled spots differed only by one additional negative charge. This was shown by defined charge mutants of chicken MM-CK [20]. Since each phosphate adds two negative charges to the protein, it is likely that both the unlabelled M-CK subspecies are getting autophosphophosphorylated which would then lead to the observed addition of one charge between each labelled spot. In order to protect the active site of CK against unspecific modifications by radiation damage, we performed the autophosphorylation reaction with a substoichiometric amount of [$\gamma$-$^{32}$P]ATP (molar ratio 1:175 of [$\gamma$-$^{32}$P]ATP/protein). Therefore the amount of incorporated radioactivity determined by PhosphoImager analysis was fairly low compared with the total amount of protein as seen in the Coomassie Blue-stained gel blots. The existence of these clearly visible CK subspecies cannot be explained by the relatively small extent of autophosphorylation alone. In fact, the Coomassie Blue stain pattern of the [$\gamma$-$^{32}$P]ATP-treated material was indistinguishable from untreated samples or an autophosphorylation incompetent mutant (results not shown). This shows that autophosphorylation cannot be the only reason for the observed microheterogeneity of MM-CK in two-dimensional IEF gels. Since we took special care to protect the protein from modifications by urea, which is present in the two-dimensional sample buffer, we can also exclude carbamylation as the cause for this heterogeneity.

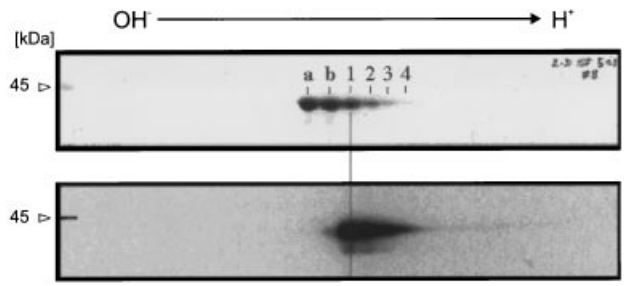


**Figure 1 *In vitro* phosphorylation pattern of MM-CK**

*In vitro* phosphorylation pattern of chicken MM-CK which was autophosphorylated with [$\gamma$-$^{32}$P]ATP and separated by two-dimensional IEF pH 8 → 5. $OH^-$, basic side; $H^+$, acidic side; the position of the 45 kDa marker (Bio-Rad, low $M_r$ standards) is shown on the left-hand side. Coomassie Blue-stained membranes (upper panel) and the corresponding autoradiography (lower panel) are also shown. Note: a phosphorylation event results in a shift by two additional negative charges towards the right-hand side.

### Chemical stability and identification of the generated phosphoamino acids

The nature of the phosphoamino-acid linkage involved in the phosphorylation was analysed by stability tests under alkaline (1 M KOH, 55 °C, 2 h) and acidic (0.15 M $HClO_4$, 55 °C, 2 h) conditions. The relative stability of the incorporated $^{32}$P-label was compared with control (0.05 M Tris, pH 8, 0.1 M NaCl,

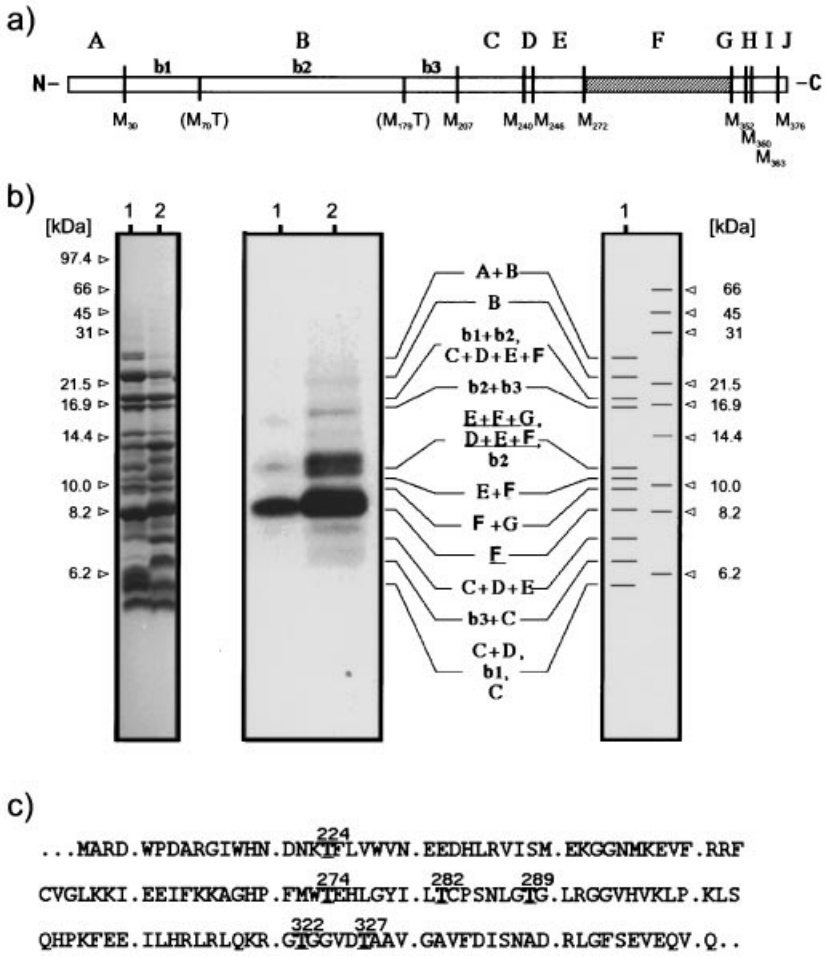


**Figure 3 Analysis of the CNBr digest of $^{32}$P autophosphorylated MM-CK**

A methionine-specific digest with CNBr was used to identify the phosphorylated sites within MM-CK. (**a**) Position of methionine residues in chicken or rabbit M-CK. Numbering includes the start methionine (see [36]). (**b**) Left: Coomassie Blue-stained PVDF membrane, showing the MM-CK peptide pattern after CNBr cleavage and separation on a 16.5% Tricine/SDS gel. Lane 1, 125 $\mu$g chicken MM-CK cleaved by CNBr; lane 2, 125 $\mu$g rabbit MM-CK cleaved by CNBr. Middle: autoradiography of the PVDF membrane after CNBr cleavage. Right: peptide size of relevant cleavage products. (**c**) Position of threonine residues within the amino-acid sequence of the identified region. The amino-acid sequence is given in standard single-letter code. Note: P-Thr was identified as the only phosphoamino acid present in CK.

55 °C, 2 h). The acid stability of the phosphoamino acid(s) (88% bound $^{32}$P) indicates the presence of O-phosphates [34]. Lability under alkaline conditions (42% bound $^{32}$P) identified phosphoserine (P-Ser) and/or phosphothreonine (P-Thr) as putative phosphorylated residues. Phosphoamino acids separated from hydrolysates of autophosphorylated CK by cellulose thin-layer plates indicated P-Thr as the only product of autophosphorylation (Figure 2). No traces of other radioactively labelled amino acids could be found in the CK hydrolysates.

## Chemical cleavage of MM-CK

To identify the phosphorylated Thr residue(s), $^{32}$P-autophosphorylated chicken and rabbit MM-CK were subjected to chemical cleavage with CNBr. This methionine-specific cleavage generates two main fragments of $M_r$ 20000 and 8782 (indicated as B and F in Figure 3a) as noted earlier [35]. The observed minor bands were due to either incomplete cleavage at CNBr-sensitive methionines or due to partial cleavage at CNBr-resistant methionine–threonine bonds [30], of which two are present within fragment 'B' (Figure 3a). Only fragment 'F' was found to be strongly labelled after $^{32}$P autophosphorylation (Figure 3b). In addition, a doublet of minor labelled bands could be assigned due to their size, to partial cleavage products containing fragment 'F'. Phosphorylated peptides containing fragment 'B' or fragments smaller than about 4 kDa were never observed, even after extensive overexposure. This clearly indicates that the main phosphorylation site(s) are located within fragment 'F' spanning residues 273–352. A similar region (amino acids 271–313), identified by different means, was reported for chicken BB-CK to contain the phosphorylation sites [13]. Fragment 'F' contains five threonine residues, which might serve as autophosphorylation target sites: Thr-274, -282, -289, -322 and -327 (Figure 3c).

Four of these threonine residues (Thr-282, -289, -322 and -327) are absolutely conserved among all CK isoforms (for detailed sequence comparison see [36]). Thr-274 is specific for chicken M-CK and conservatively replaced by asparagine (Asn) in other CKs. A minor putative phosphorylation site in fragment 'E', Thr-224, is conserved in all BB-CKs but replaced by a serine in most of the M-CKs (except chicken and dog).

**Table 1 Extents of autophosphorylation, Michaelis–Menten constants and specific activities of the different chicken MM-CK threonine-replacement mutants**

The autophosphorylation steady-state levels are given as relative values with respect to the wild-type (wt) chicken MM-CK, which was defined as 100%. The kinetic constants of the different proteins were determined using the pH-STAT assay [49]. For the determination of the $K_m$ values for PCr, the concentration of ADP was kept constant at 4 mM and PCr was varied between 0.25 and 50 mM, with some mutant enzymes up to 100 mM PCr. For the determination of the $K_m$ values for ADP, the concentration of PCr was kept constant at 10 mM and ADP was varied between 0.1 and 4 mM, with some mutant enzymes up to 10 mM ADP. n.d., not determined.

| Enzyme | Relative extent of autophosphorylation (%) | $K_m$ (mM) | | Forward reaction | | Reverse reaction | |
|---|---|---|---|---|---|---|---|
| | | ADP | PCr | units/mg | wt% | units/mg | wt% |
| Chicken MM-CKwt | 100 | 0.16 ± 0.011 | 1.76 ± 0.179 | 26.90 | 100 | 200 | 100 |
| T224V | 81 ± 3.9 | 0.14 ± 0.011 | 2.71 ± 0.195 | 19.30 | 71.8 | 145 | 72.5 |
| T274V | 48 ± 1.3 | 0.16 ± 0.009 | 1.79 ± 0.116 | 6.83 | 25.4 | 58.3 | 29.1 |
| T282V | 32 ± 2.7 | 0.14 ± 0.011 | 2.59 ± 0.288 | 13.60 | 50.6 | 83.2 | 41.6 |
| T289V | 21 ± 1.4 | 0.51 ± 0.028 | 2.68 ± 0.269 | 4.95 | 18.4 | 96.7 | 48.3 |
| T322V | 25 ± 2.1 | 0.15 ± 0.010 | 4.04 ± 0.814 | 7.81 | 29.1 | 69.3 | 34.7 |
| T327V | 27 ± 1.5 | 0.25 ± 0.029 | 7.33 ± 0.768 | 8.00 | 29.8 | 98.3 | 49.2 |
| T274,289V | 13 ± 1.6 | 0.94 ± 0.049 | 5.64 ± 0.518 | 3.62 | 13.5 | 93.3 | 46.7 |
| T282,289V | 17 ± 2.5 | 0.44 ± 0.032 | 2.58 ± 0.115 | 2.40 | 8.94 | 48.0 | 24.0 |
| T322,327V | 24 ± 8.5 | 0.12 ± 0.014 | 15.24 ± 0.514 | 1.72 | 6.39 | 18.1 | 9.07 |
| T274,282,289V | 6 ± 0.9 | 0.77 ± 0.036 | 6.39 ± 0.452 | 0.02 | 0.06 | 1.23 | 0.62 |
| T289,322,327V | 15 ± 1.3 | 0.44 ± 0.031 | 15.14 ± 0.267 | 0.15 | 0.56 | 9.09 | 4.55 |
| T224,274,282,289V | 37 ± 4.8 | n.d. | n.d. | 0 | 0 | 0.02 | 0.01 |
| T224,274,282,289,322,327V | 5 ± 3.9 | n.d. | n.d. | 0 | 0 | 0.02 | 0.01 |

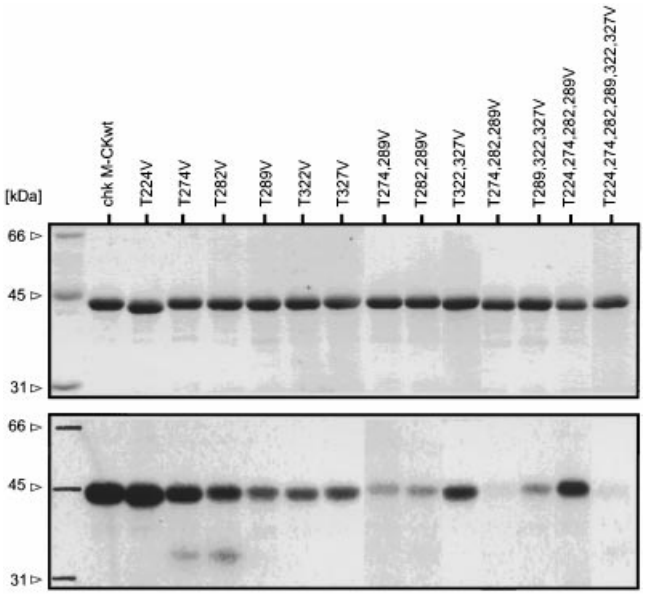


**Figure 4 Autophosphorylation analysis of threonine-replacement mutants**

Wild-type chicken MM-CK and defined threonine mutants thereof, expressed and purified from *E. coli*, were autophosphorylated under standard conditions. Identical protein amounts, each 3 $\mu$g, were subsequently separated on 11% SDS/PAGE. The corresponding mutant proteins are indicated in a descriptive manner on the top. Upper panel: Coomassie Blue-stained SDS/PAGE. Lower panel: corresponding autoradiography.

### Threonine-replacement mutants

All threonine residues that represent putative autophosphorylation sites were substituted with valine, either as a single threonine–valine-replacement mutant or as a variety of multiple mutants, by different combinations thereof. The effects of the amino-acid substitutions on the autophosphorylation potential and enzymic activity were analysed with purified mutant enzymes (Table 1).

For a quantitative analysis of the autophosphorylation in these recombinant mutant proteins, identical amounts of protein were simultaneously autophosphorylated in identical reactions, separated by gel electrophoresis and subsequently quantified by phosphorimager analysis (Figure 4).

All threonine mutants showed a substantially decreased autophosphorylation rate, as compared with the wild type (Table 1). Out of the single mutants, the replacement of the highly conserved residues (Thr-282, -289 -322 and -327) had a much stronger effect than replacing the chicken M-CK-specific Thr-274. However, no single threonine mutant abolished autophosphorylation completely, indicating that somehow all six threonines take part in this process. Only when all six threonines were removed together was autophosphorylation almost abolished.

The specific activities of the different mutant enzymes, for the forward and reverse reactions, are compiled in Table 1. Most mutants showed a decreased but significant enzymic activity, with the exception of those mutants in which four Thr residues (Thr-224, -274, -282 and -289) were exchanged altogether. With this quadruple mutant, only a minute residual activity was measured in the reverse reaction, whereas the forward reaction was below the detection limit. Generally, the more threonines exchanged, the less activity recovered, with forward and reverse reactions being affected in a similar way. Only proteins containing the T289 mutation consistently displayed a much more reduced specific activity in the forward reaction compared with that of the reverse reaction.

The $K_m$ showed site-specific effects of the Thr to Val mutation on the substrate binding. The exchange of Thr-289 for a valine caused a consistent 3.2–5.8-fold increase of the $K_m$ for ADP. This was most pronounced in combination with T274V, which seems to have a co-operative effect. A negligible increase in $K_m$ (ADP) could also be observed with T327V.

The kinetic analysis also indicated that Thr-322 and Thr-327 are key residues in determining the $K_m$ for PCr with T327V showing a stronger effect than T322V.

Interestingly, there was no direct and consistent correlation between the determined autophosphorylation rates of the different threonine mutants and the enzymic activity. Furthermore, autophosphorylation ultimately did not depend on the catalytic

activity (see Table 1). For instance, the relative autophosphorylation of the mutant T322,327V (24 %) was very similar to that in some of the single mutants, whereas the $K_m$ for PCr showed a several-fold increase compared with the other mutants or the wild type. Similarly, mutant T274,289V showed a 5.8-fold increase in the $K_m$ for ADP, whereas the relative extent of autophosphorylation (13 %) was similar to other mutants with an inconspicuous $K_m$ for ADP. Most pronounced for this discrepancy was the 4-fold mutant T224,274,282,289V, which showed basically no residual enzymic activity but a relatively high extent of autophosphorylation, which can be assigned mainly to T322 and T327.

## DISCUSSION

Autophosphorylation is a phenomenon that has been demonstrated to take place in all CK isoforms [13,37,38]. Recently, this autophosphorylation of CK has been challenged and it was claimed that the enzyme is only nucleotidylated, instead [15]. This finding was not confirmed by our experiments. Using [$\gamma$-$^{32}$P]ATP we could unambiguously show that the $\gamma$-$^{32}$P is covalently linked to the protein by autophosphorylation. After hydrolysis of [$\gamma$-$^{32}$P]-treated MM-CK, P-Thr was the only radioactively labelled amino acid that could be found (Figure 2).

This discrepancy in the results might be caused by slightly different experimental conditions. Whereas David and Haley [15] carried out their experiments in the absence of reducing agents, we had 2 mM DTT present during the autophosphorylation reaction. Reducing agents, such as $\beta$-mercaptoethanol or DTT, have been shown to protect the enzyme efficiently against radiation and radical damage within the active site [39]. This is especially true for the reactive active-site Cys-283 which was claimed to be the target of the nucleotidylation reaction. The radiation dosage which David and Haley [15] used during their experiments was in the range $\approx$ 100–200 Gy. This dosage is known to be sufficient to introduce severe radiation damage, especially if concentrated in the form of radioactive ATP within the active site. Such radiation damage was shown to cause a significant inactivation ($D_{37} \approx 115$ Gy for MM-CK) of the CK enzyme [39]. Although we do not exclude the possibility that nucleotidylation of CK may occur *in vivo*, it seems possible that the observed *in vitro* nucleotidylation is prone to artifacts caused by the radiation dosage used. The nucleotide might be cross-linked into the active site by the action of free radicals generated by radiation, especially in the absence of sulphydryl-protecting agents like DTT or $\beta$-mercaptoethanol.

In our experiments, $^{32}$P-labelled material represents only a small fraction of the protein spots that are observed as microheterogeneity on IEF gels after Coomassie Blue stain. Since the 6-fold mutant, which basically lacks the ability for autophosphorylation (see Table 1), does still show an indistinguishable protein microheterogeneity pattern (results not shown), it is clear that the observed pattern cannot be exclusively caused by phosphorylation events. The origin of the observed microheterogeneity is probably an artifact, caused either by pH-dependent conformational transitions and/or by interactions of CK with the carrier ampholytes in the two-dimensional IEF gel [27].

We confirmed that distinct threonine residues in the active site of chicken MM-CK are the prime target sites for the autophosphorylation mechanism. Their substitution with valine resulted consistently in a substantial decrease in the extent of autophosphorylation. However, we could not identify a single defined threonine residue, which serves as an exclusive phosphorylation target. Instead, multiple threonine-specific target sites were found for the autophosphorylation mechanism present in CK. Interestingly, this autophosphorylation mechanism is not dependent on CK activity. This was corroborated by mutant T224,274,282,289V which showed basically no catalytic activity but still a relatively high extent of autophosphorylation (37 % compared with the wild type). This is in agreement with earlier findings, showing that enzyme inactivation with dinitrofluorobenzene which reacts specifically with the reactive sulphydryl group of Cys-283 [40], does not inhibit autophosphorylation of the enzyme [14]. This means that the observed autophosphorylation is not a direct result of the CK transphosphorylation reaction.

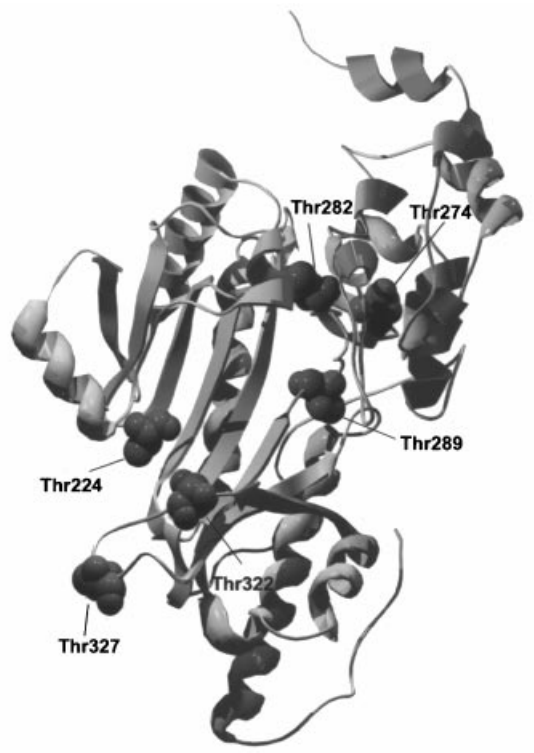


**Figure 5 Localization of the identified threonine residues in the CK structure**

The threonine positions, identified as autophosphorylation target sites, are shown in the three-dimensional X-ray structure of B-CK. Thr-322 and Thr-327 are located on a highly flexible loop and influence the PCr affinity of the enzyme. Thr-289 is located close to the active site in the P-loop region and seems to be involved in the affinity towards the nucleotide substrate. The highly homologous B-CK structure [47] was used instead of the M-CK structure [48] because of its higher resolution and better localization of the flexible-loop region. Note that M-CK-specific Thr-274 is replaced by an asparagine in B-CK.

Two of the identified threonine residues are of particular interest because of their position within the active site of CK (Figure 5). Thr-282 is located just next to the highly reactive Cys-283. Although it was concluded that this reactive cysteine is not directly involved in substrate binding [41], it seems to be responsible for the correct orientation of the guanidino substrate within the active site [42]. Thus a phosphorylation at this position would be located at a strategically very sensitive position to potentially influence the enzyme activity. The kinetic data indicate that Thr-289 seems to be a key residue for the binding of ADP and further evidence suggests that this residue is even more important for the binding of ATP. Thr-289 is located in a glycine-rich-motif ($L_{287}$-G-T-G-L-R-G-G-$V_{295}$), very similar to the consensus sequence (L-G-X-G-X-X-$G_{(A,S)}$-X-V) of the phos-

phate-binding loop (P-loop) originally found in protein kinases [43,44]. This region is thought to be involved in the anchorage of the $\beta$- and $\gamma$-phosphates of the bound ATP and also in the co-ordination of $Mg^{2+}$, when a metal ion is required [36].

Both Thr-327 and Thr-322 could be shown to be important for the binding of PCr. The location of these residues in the flexible-loop region (residues 321–331) imply a conformational change in this region, to bring this loop into the vicinity of the $\gamma$-phosphate of the bound nucleotide, as shown also by the observed kinetic effects (Figure 5).

The extent of autophosphorylation is not directly correlated with the changes in the different kinetic parameters. However, the data allowed us to suggest that the autophosphorylation rate and specificity depend on the correct positioning of the substrate, i.e. they are directly influenced by the structural disturbances around the phosphate tail in the different mutants. This explains why EDTA can abolish the autophosphorylation in a concentration-dependent manner [14]. It was thought that only the $Mg^{2+}$-ion-co-ordinated adenine nucleotides (MgADP or MgATP) can introduce structural changes essential for catalysis by orienting several key residues in the active site [45]. The $Mg^{2+}$-ion-co-ordinated nucleotide adopts a more bent conformation, whereas the free nucleotide has an elongated one. Thus the removal of $Mg^{2+}$ would result in a structural impairment not only of the nucleotide but also of the active site. These observations lead to the conclusion that the CK autophosphorylation mechanism controlled by structural constraints is involved in the correct orientation of MgATP. The modification mechanism itself is highly specific for threonine residues in the vicinity of the active site, but it is not selective for a single threonine. The participation of the identified threonine residues in the substrate binding implies a functional role for phosphorylation in the recognition of the substrate. In this context, it is interesting that all mutants which were modified at Thr-289 showed a lowered affinity for the nucleotide, but this decreasing effect was not equivalent in both directions of the reaction. The forward reaction (PCr production) was far more inhibited than the reverse reaction towards ATP synthesis. So, CK autophosphorylation may possibly modulate the reversibility of the CK reaction. A similar regulatory control by phosphorylation was reported for enolase, an enzyme catalysing a reversible step in the glycolytic pathway [46].

However, at the moment it is not clear whether CK autophosphorylation is a directionless self-affinity labelling, as a by-product of the intimate vicinity of the threonines to the bound MgATP, or a regulatory mechanism, controlled by factors like changes in pH or a low-substrate stoichiometry. Elucidation of the putative control mechanisms for CK autophosphorylation still remains a subject for future studies.

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