Creatine Kinase: The Reactive Cysteine Is Required for Synergism But Is Nonessential for Catalysis†

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ABSTRACT: Chemical modification of rabbit muscle creatine kinase (CK) with thiol-specific reagents led to partial or complete inactivation of the enzyme. Using site-directed mutagenesis, we have substituted the corresponding reactive Cys278 in the chicken cardiac mitochondrial creatine kinase (Mi_b-CK) with either glycine, serine, alanine, asparagine, or aspartate. The resulting mutant Mi_b-CK enzymes showed qualitatively similar changes in their enzymatic properties. In both directions of the CK reaction, a shift of the pH optimum to lower values was observed. Mutant Mi_b-CKs were severalfold more sensitive to inhibition by free ADP in the reverse reaction (ATP synthesis) and to free ATP in the forward reaction (phosphocreatine synthesis). With the exception of C278D, all mutant enzymes were specifically activated by chloride and bromide anions. C278D and wild-type Mi_b-CK were significantly inhibited under the same conditions. At low chloride concentrations, the V_{max} of C278D was about 12-fold higher than that of C278N. Thus, Cys278 probably provides a negative charge which is directly or indirectly involved in maximizing CK activity. Under near-optimal conditions in the reverse reaction, mutants C278G and C278S showed about an 11-fold increase in K_m(PCr), but only 1.7- and 2.8-fold reductions in V_{max}, respectively, compared to wild-type Mi_b-CK. Thus, the reactive cysteine clearly is not essential for catalysis. For rabbit muscle CK, substrate binding had been shown to be synergistic (i.e., $K_d > K_m$). We confirmed this finding for wild-type Mi_b-CK by determining the K_d and K_m values for both substrates in the forward reaction. Analysis of these constants for the two mutant enzymes C278G and C278S showed that the reactive cysteine (1) is not directly involved in binding either substrate (K_d values for mutants were not dramatically changed compared to the wild type) and (2) is necessary for synergistic substrate binding (K_d values for mutants were smaller than the corresponding K_m values). These results suggest that the reactive cysteine is necessary to confer conformational changes upon substrate binding and support the proposal that this residue has a role in shaping the active site, possibly by acting as a hinge between the two substrate binding sites.

One important enzyme in cellular energy metabolism is creatine kinase (CK;[1] EC 2.7.3.2), which reversibly transfers a phosphoryl group from ATP to creatine. The enzyme exists in different isoforms which are encoded by individual genes. The expression of the isoforms is regulated in a temporal and tissue-specific manner, and the subcellular localization is also isoform specific [for a recent review, see Wallimann et al. (1992)]. The "cytosolic" M- and B-CK isoenzymes form homo- and heterodimers (Eppenberger et al., 1964). Two additional isoforms are restricted to the mitochondria (Jacobs et al., 1964; Jacobus & Lehninger, 1973), where they exist in an octameric form, consisting of four dimeric subunits, with a total molecular weight of about 340 000 (Wyss et al., 1992). Despite the isoform-specific patterns of expression and localization, the amino acid sequences deduced from cDNAs for different CK isoenzymes from more than 20 sources are highly conserved from sea urchins to mammals [for references, see James et al. (1990)].

Although there is no X-ray crystallographic structure available yet for CK, a wealth of biochemical and spectroscopic information regarding kinetic and mechanistic aspects of the CK reaction has been produced and extensively reviewed (Watts, 1973; Kenyon & Reed, 1983). Several amino acid residues have been implicated through detailed studies to play a role in substrate binding, including arginine (Borders & Riordan, 1975; James, 1976), lysine (Kassab et al., 1968; James & Cohn, 1974), tryptophan (Vasák et al., 1979), and aspartate (James et al., 1990). A histidine residue has been proposed to act as an acid/base catalyst which protonates/deprotonates the phosphate-accepting nitrogen of creatine (Cook et al., 1981; Rosevear et al., 1981). So far, none of these residues has been localized in the primary sequence.

One highly reactive sulfhydryl group per protomer has been found in all analyzed creatine kinases. Cytosolic isoforms chemically modified with several sulfhydryl-specific reagents were completely inactive [Mahowald et al., 1962; reviewed in Kenyon and Reed (1983)], but a few reagents tested allowed residual activity (Smith & Kenyon, 1974; der Terrossian & Kassab, 1976; Maggio et al., 1977). However, these latter findings have been challenged more recently by extensive studies suggesting that the reactive sulfhydryl group of CK is indeed essential for catalysis (Reddy & Watts, 1979; Wu et al., 1989; Zhou & Tsou, 1987). Inactivation of the mitochondrial isoforms with sulfhydryl-specific reagents was

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[1] Abbreviations: Ac, acetate anion; CK, creatine kinase; B-CK, brain-type CK; M-CK, muscle-type CK; Mi-CK, mitochondrial-type CK isoforms; Mi_b-CK, basic, "sarcomeric" Mi-CK isoform; PCr, phosphocreatine; Cr, creatine. Mutants with amino acid substitutions are denoted in the standard one-letter code by the wild-type residue and numbered position within the sequence, followed by the amino acid substitution. Thus, C278G Mi_b-CK is a mutant enzyme with a glycine residue at position 278 instead of the cysteine. K_d refers to the dissociation constant of the reaction E + A → EA or E + B → EB, and K_m refers to the Michaelis–Menten constant of the reaction EA + B → EAB or EB + A → EAB, whereby A and B represent either ADP and PCr or ATP and Cr, respectively. Forward reaction is PCr synthesis; reverse reaction is ATP synthesis.

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also observed (Fedosov & Belousova, 1988; Wyss et al., 1993). The reactive cysteine residue has been identified as Cys282 in rabbit M-CK, corresponding to Cys278 in the chicken Mi-CK isoenzymes, which are shorter by four residues at their N-termini. This cysteine residue is conserved in all known CK sequences.

The role of the reactive cysteine in catalysis and substrate binding is still not well understood and remains controversial [for a recent discussion, see Buechter et al. (1992)]. To further elucidate the function of this residue, we have substituted the reactive Cys278 in the chicken cardiac mitochondrial creatine kinase (Mi_b-CK) by site-directed mutagenesis. Mutant enzymes have been expressed in *Escherichia coli* and were characterized extensively after purification. The kinetic properties of the different mutant enzymes corroborate and clarify many of the earlier findings and conclusions which were based on sulfhydryl-modified rabbit muscle CK. We have shown that the reactive cysteine is not essential for substrate binding or for catalysis. However, this residue plays a dual role in the CK reaction: it is required for synergistic substrate binding, and it provides a negative charge which is necessary for maximum enzyme activity. Since both the primary structure and the function are highly conserved among all CK isoenzymes, the knowledge gained with Mi_b-CK can most likely be transferred to other CK isoenzymes. These results will also assist in defining the boundaries of the active site once an X-ray structure of CK is available.

MATERIALS AND METHODS

Protein Preparation. Growth media for *Escherichia coli* and the methods for standard DNA manipulation have been described (Sambrook et al., 1989). *E. coli* strain BL21(DE3)-pLysS and expression vector pET-3b (Studier et al., 1990) were used for the expression of Mi_b-CK wild-type and mutant proteins, as has been described in detail for the wild-type Mi_b-CK enzyme (Furter et al., 1992). Amino acid replacements of the conserved cysteine at position 278 in the chicken Mi_b-CK cDNA were introduced by site-directed mutagenesis (Nakamaye & Eckstein, 1986). The complete coding sequence of each individual mutant allele was checked by dideoxy sequence analysis of the double-stranded expression plasmid.

Protein expression and purification were performed as has been described earlier (Furter et al., 1992). In short, freshly transformed BL21(DE3)pLysS cells were grown at 37 °C to an A_{590} of about 0.7 in aliquots of 375 mL of 2XYT medium containing 100 mg/L ampicillin. Expression of Mi_b-CK protein was then induced by the addition of 0.4 mM β-D-thiogalactopyranoside (IPTG). After an additional 4–5 h of growth, cells were harvested, washed, and lysed by sonication. All Mi_b-CK mutant proteins with amino acid substitutions at position 278 were expressed in soluble form, and the amount of expressed mutant protein was comparable to the amount of wild-type Mi_b-CK protein. The soluble fraction of the *E. coli* extract was enriched for Mi_b-CK protein by column chromatography on Blue Sepharose CL-6B and by FPLC chromatography on Mono S HR5/5 (Pharmacia). Routinely, 20–50 mg of nearly homogeneous protein was obtained from 1 L of *E. coli* culture, representing an overall yield of about 50% and an approximately 3-fold enrichment, as found earlier for the wild-type protein expressed in the same way (Furter et al., 1992). Protein concentrations were determined with the Bio-Rad protein assay (Bradford, 1976) with BSA as the standard. The oligomeric pattern of Mi_b-CK was characterized by FPLC Superose 12 HR 10/30 gel permeation chromatography as has been described (Schlegel et al., 1988).

Enzyme Activity Assays. Creatine kinase activities were determined by the pH–stat method by measuring the H^+ release in the forward reaction, creatine + ATP → phosphocreatine + ADP + H^+, and the H^+ consumption in the reverse reaction, phosphocreatine + ADP + H^+ → creatine + ATP, as has been described earlier (Milner-White & Watts, 1971; Wallimann et al., 1984). Reaction conditions for the individual experiments are described in detail in the text or in the appropriate figure or table. At pH values below 6.0, PCr transphosphorylation and H^+ consumption are no longer equimolar. Correspondingly, the pH–stat values were corrected for this discrepancy, on the basis of calculated correction factors and experimental calibrations (Wyss, 1992).

For the determination of constants K_m and K_d, inhibition constants, and V_{max}, initial velocity data were analyzed using the program package written by W. W. Cleland (Cleland, 1979) as adapted for personal computer by R. Viola (the program was obtained through R. Viola, Akron University, Akron OH). Standard errors are given for the calculated values unless otherwise stated. Each set of constants represents the mean of three or four series of independent measurements.

Calculation of Free ADP Concentrations. For the determination of inhibition constants for free ADP, it was necessary to calculate the concentrations of free and magnesium-bound ADP. These concentrations are dependent on the concentrations of added Mg^{2+}, ADP, PCr, and H^+. The complex formed between Mg^{2+} and PCr, a minor species in the assay, was neglected for all calculations. For the apparent stability constant of $MgADP^-$, a value of 4000 (at pH 8.0) was used. For calculations, the formation of MgHADP from Mg^{2+} and $HADP^{2-}$ was taken into account with an apparent stability constant of 100 (O'Sullivan & Smithers, 1979). Experimentally, a constant [$MgADP^-$] of 1 mM was chosen. The $[ADP^{3-}]_{free}$ was varied between 0.05 and 6 mM. To achieve this, the $[Mg^{2+}]_{total}$ and $[ADP]_{total}$ had to be varied, also leading to variable $[Mg^{2+}]_{free}$ and $[HADP^{2-}]_{free}$. Any possible inhibitory effects of these additional ligands were neglected.

RESULTS

Expression, Purification, and Structural Analysis of Mi_b-CK Mutant Proteins. The highly reactive Cys278 in the mitochondrial creatine kinase (Mi_b-CK) was substituted with either a Gly, Ser, Ala, Asn, or Asp residue. All mutant proteins and the wild-type protein were expressed in *E. coli* with the help of the T7-phage promoter in the expression vector pET-3b (Studier et al., 1990). All of the mutant forms with changes at position 278 were equally well expressed as the wild-type Mi_b-CK. Each mutant protein was obtained in soluble form and could be purified following the protocol developed for the wild-type Mi_b-CK (Furter et al., 1992). In particular, the elution profiles of mutant and wild-type Mi_b-CK proteins from the FPLC Mono S column were identical (not shown). The mutant proteins were also equally stable during extended storage at 4 °C. These observations indicate that substitutions at Cys278 have no adverse effect on the proper folding and stability of Mi_b-CK. To further test the overall structure, the mutant proteins C278G, C278S, and C278A were analyzed by gel permeation chromatography on an FPLC Superose 12 HR 10/30 column. All mutant proteins showed an elution pattern identical to the wild-type protein (Schlegel et al., 1988), that is, more than 90% of the protein eluted as the octameric form of Mi_b-CK and the minor protein peak eluted at the position characteristic for the dimeric form (not shown). In addition, CD spectra for the wild-type and C278G Mi_b-CK were identical (M. Gross, unpublished), and preliminary X-ray

Table I: Specific Activities of Different Mi_b-CK Enzymes[a]

enzyme	forward reaction		reverse reaction, 65 mM KCl[b]		reverse reaction, without KCl[c]	
	units/mg[d]	%	units/mg[d]	%	units/mg[d]	%
wild-type	46.4 ± 2.8	100	74.1 ± 4.0	100	87.9 ± 2.5	100
C278G	0.41 ± 0.02	0.9	2.18 ± 0.19	3.0	0.22 ± 0.06	0.3
C278S	0.18 ± 0.02	0.4	0.81 ● 0.07	1.1	0.21 ± 0.05	0.2
C278A	0.036 ± 0.008	0.08	0.179 ● 0.016	0.24	0.024 ± 0.002	0.03
C278D	nd[e]		0.042 ± 0.011	0.06	0.062 ± 0.002	0.07
C278N	nd		0.044 ± 0.006	0.06	0.030 ± 0.011	0.03

[a] Specific enzyme activities (±standard deviation) averaged for two independent enzyme preparations are shown. Activities were reproducible between individual preparations. The specific activities for the forward reaction (Cr + ATP → PCr + ADP + H^+) were determined under the following conditions: 90 mM Cr, 17.5 mM ATP, 17.5 mM $MgAc_2$, 65 mM KCl, and 1 mM 2-mercaptoethanol at pH 8.4, 25 °C. [b] Specific activities in the reverse reaction (PCr + ADP + H^+ → Cr + ATP) were determined under the following conditions: 10 mM PCr, 4 mM ADP, 8.5 mM $MgCl_2$, 65 mM KCl, and 1 mM 2-mercaptoethanol at pH 7.0, 25 °C. [c] In the absence of KCl, the conditions were 10 mM PCr, 4 mM ADP, 10 mM $MgAc_2$, and 1 mM 2-mercaptoethanol at pH 7.0, 25 °C. [d] 1 unit of enzyme activity is equal to 1 μmol of ATP or PCr transphosphorylated per minute, respectively, at 25 °C. [e] nd, not determined.

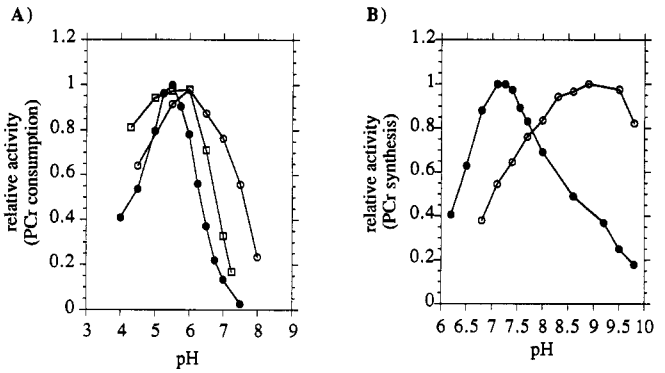

FIGURE 1: pH optimum curves for wild-type and mutant Mi_b-CK enzymes. Relative activities (ratio of initial rates) in dependence of pH are shown. The maximum activity for each Mi_b-CK enzyme was set at the nominal value of 1; however, note that the absolute specific activities of all mutants are lowered compared to that of the wild-type Mi_b-CK. (A) Reverse reaction (ATP synthesis). Reaction conditions were 10 mM PCr, 1 mM ADP, 8.5 mM $MgCl_2$, 65 mM KCl, and 1 mM 2-mercaptoethanol at 25 °C. Activities were corrected for the nonstoichiometrical relation between H^+ and PCr consumption at pH values below 6.0. ○, wild-type Mi_b-CK. ●, C278G and C278S exhibited identical curves within experimental error. □, C278N and C278D exhibited similar curves. (B) Forward reaction (PCr synthesis). Reaction conditions were 45 mM Cr, 5.4 mM ATP, 7.4 mM $MgAc_2$, 65 mM KCl, and 1 mM 2-mercaptoethanol at 25 °C. ○, wild-type Mi_b-CK. ●, C278G and C278S exhibited identical curves within experimental error.

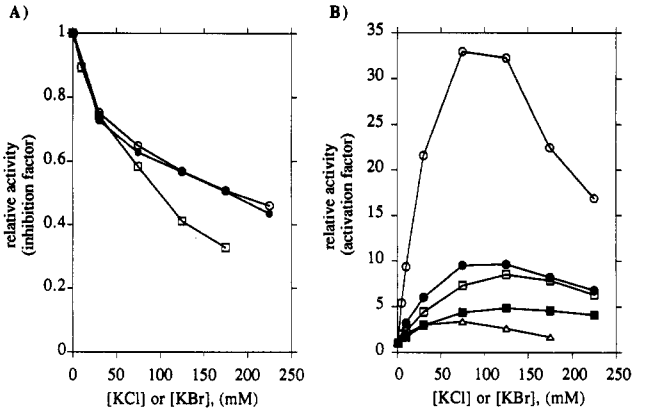

FIGURE 2: Influence of KCl and KBr on the activity of wild-type and mutant Mi_b-CK enzymes. Relative activities (ratio of initial rates) in dependence of KCl and KBr concentrations, respectively, in the reaction mixture are shown in the reverse reaction (ATP synthesis). Activities in the absence of Cl^- or Br^- ions are set at 1. Reaction conditions were 10 mM PCr, 1 mM ADP, 10 mM $MgAc_2$, and 1 mM 2-mercaptoethanol at pH 6.0, 25 °C. (A) ○, activity of wild-type Mi_b-CK with increasing KCl; ●, activity of wild-type Mi_b-CK with increasing KBr; □, activity of C278D with increasing KCl. (B) ○, activity of C278G with increasing KCl; ●, activity of C278G with increasing KBr; □, activity of C278S with increasing KCl; ■, activity of C278S with increasing KBr; △, activity of C278N with increasing KCl.

data confirmed that C278G crystals display the same space group and unit cell dimensions as wild-type crystals (T. Schnyder, unpublished). The Superose profiles confirm the intact oligomeric structure of the enzyme, and the CD spectra show that no obvious change in the secondary structure has been introduced by the substitution of the cysteine residue.

Enzyme Activity. As can be seen from Table I, the specific activities of all of the mutant Mi_b-CKs were drastically reduced in both directions of the CK reaction, employing a standard assay mixture. However, all of the mutant enzymes still exhibited some activity, indicating that the Cys278 is not essential for the enzyme activity of Mi_b-CK. The assay conditions used for the measurements presented in Table I were originally established for the wild-type enzyme. To evaluate the observed differences in enzymatic activity between wild-type and mutant Mi_b-CK, the optimal reaction conditions first had to be established. The initial velocity as a function of pH in the reaction mixture was determined for the forward and reverse reactions. In the reverse (Figure 1A) as well as in the forward reaction (Figure 1B), the mutant enzymes showed a shift of the pH curve to a lower pH optimum. The observed shift in pH optimum depicted in Figure 1A was corroborated by the determination of maximal activities (V_{max}) and K_m values at three different pH values for the reverse reaction (see Table III) for the wild-type and the C278G mutant enzyme. These values clearly show that the wild-type enzyme has a higher pH optimum than the C278G mutant enzyme. We made use of this observation by routinely measuring initial velocities at pH 6.0 rather than at pH 7.0, as originally used for the wild-type enzyme in earlier works.

Muscle-type CK is partially inhibited by Cl^- ions (Milner-White & Watts, 1971). To assess the influence of salt conditions on the activity of the wild-type and mutant Mi_b-CKs, activity measurements were performed in the reverse reaction with increasing salt concentrations; in addition, different anions and cations were tested. The wild-type Mi_b-CK was progressively inhibited by increasing KCl or KBr concentrations (Figure 2A). Almost identical inhibition was caused by NaCl, NaAc, LiCl, and KI (not shown). C278D Mi_b-CK was even a bit more sensitive than the wild-type enzyme to inhibition with KCl (Figure 2A). Surprisingly, the activity of the other mutants responded completely differently (Figure 2B). Cl^- ions activated C278G (33-fold), C278S (8.5-fold), and C278N (3.4-fold). Of the different salts tested, KCl, NaCl, and LiCl showed almost identical activation (not shown). KBr activated C278G and C278S to

Table II: Michaelis–Menten Constants for ATP Synthesis (Reverse Reaction) at Different KCl Concentrations

	65 mM KCl		3 mM KCl	
enzyme	K_m(PCr) (mM)	V_{max} (units/mg)[c]	K_m(PCr) (mM)	V_{max} (units/mg)[c]
wild-type[a]	1.21 ± 0.05	78.3 ± 1.1	0.68 ± 0.02	98.4 ± 1.1
C278G[a]	23.5 ± 0.8	7.9 ± 0.2	16.3 ± 1.4	0.43 ± 0.02
C278S[a]	14.4 ± 0.9	2.2 ± 0.02	7.1 ± 0.9	0.29 ± 0.02
C278D[b]	32.5 ● 1.5	0.94 ± 0.03	8.7 ± 0.7	1.05 ± 0.03
C278N[b]	18.0 ± 0.9	0.61 ± 0.02	10.2 ± 2.3	0.09 ± 0.01

[a] For the wild-type Mi_b-CK and indicated mutants, assay mixtures containing 65 mM KCl also contained 4 mM ADP, 8.5 mM $MgCl_2$, and 1 mM 2-mercaptoethanol at pH 7.0, 25 °C; assay mixtures with 3 mM KCl also contained 4 mM ADP, 10 mM $MgAc_2$, and 1 mM 2-mercaptoethanol at pH 7.0, 25 °C. [b] Assay conditions for the indicated mutants were 1 mM ADP, 10 mM $MgAc_2$, and 1 mM 2-mercaptoethanol at pH 6.0 and 25 °C, with either 65 or 3 mM KCl. [c] For unit definition see Table I. PCr concentrations were varied between 30 and 0.2 mM for the wild-type enzyme and between 30 and 3 mM for the mutant enzymes. Values determined for the enzymes under the conditions in footnote a are not directly comparable to those in b because of the pH dependence of activity and different concentrations of Mg^{2+} and ADP. Mutant enzymes are more sensitive to inhibition by free ADP (see also in the text below and Table V). Kinetic parameters shown in Table III are not directly comparable to values in Table II since the assay mixture used for Table III contained lower free ADP concentrations.

a lesser extent (Figure 2B), and KI showed only very weak activation (not shown). No activation was observed with KNO_2, KNO_3, $NaSO_4$, NaAc, $(NH_4)Ac$, $NaClO_4$, and NaF (not shown). Thus, the salt activation of the mutant enzymes showed a rather high preference for Cl^-. We also observed very similar Cl^- activation at pH 7.0 (not shown). In the forward reaction, the activation factors were smaller than in the reverse reaction, but the Cl^- specificity of activation was maintained (not shown). Since high salt concentrations increase the K_m value for PCr to some extent (see Table II), the activating effect of Cl^- is not due to a better substrate saturation of the enzymes at 65 mM KCl. More likely, the salt activation is directly influencing the catalytic rate.

The mutant pair C278D and C278N is particularly informative about the role of the activating Cl^- ions. The main difference in these two substitutions is the negative charge that is provided by the Asp278 residue. The Asp-containing mutant enzyme is inhibited by Cl^- (Figure 2A), while the C278N mutant, lacking the negative charge, is activated by Cl^- anions (Figure 2B). This conclusion is further substantiated by comparing the V_{max} values of these mutants determined at 65 and 3 mM KCl, respectively (see Table II).

K_m and V_{max} Determination for ATP Synthesis (Reverse Reaction). The optimal salt and pH requirements of the mutant Mi_b-CK enzymes were greatly altered compared with the conditions for the wild-type enzyme. To show whether salt and/or pH influenced substrate binding and/or catalysis, the Michaelis–Menten constants V_{max} and K_m for PCr were determined. It was not possible to determine accurately the K_m value for MgADP with the pH–stat method for technical reasons, since this K_m is well below 100 μM [for a review, see Wyss et al. (1992)]. There is no indication from our work, however, that K_m values for MgADP were substantially increased for any of the mutants. Therefore, the K_m values for PCr were determined at a constant MgADP concentration of 1 mM.

Under all conditions tested, the K_m(PCr) values were increased in all of the mutant enzymes, and the maximal activities (V_{max}) were reduced compared with the wild-type enzyme (see Tables II and III). For all of the Mi_b-CK enzymes, K_m(PCr) was increased by a higher KCl concentration (Table II). With the exception of the C278D and wild-type Mi_b-CKs, all of the Mi_b-CKs had a severalfold higher V_{max} in the presence of 65 mM KCl than with 3 mM KCl. At 3 mM KCl, the V_{max} of mutant C278D is about 12-fold higher than the V_{max} of C278N. At 65 mM, the V_{max} values of C278D and C278N Mi_b-CK are similar but are clearly lower than for C278G and C278S (Table II). This behavior strongly suggests that, in the absence of chloride ions, a charged amino acid side chain at position 278 mimics the role of the cysteine residue better than an uncharged residue of very similar size.

For both the wild-type and the mutant enzymes, a decrease in the pH led to a decrease in K_m(PCr) (Table III). V_{max} values were highest at the pH values where maxima for specific activities were seen in pH titration experiments (see Figure 1A), providing corroboration for the shift in pH optima being not only an apparent shift due to changed K_m values. Strikingly, the V_{max} values of the C278G and C278S Mi_b-CKs under near-optimal conditions (pH 5.5, 1 mM ADP, 15 mM $MgCl_2$, 65 mM KCl, and 1 mM 2-mercaptoethanol; see Table III) were reduced only 1.3- and 2-fold, respectively, compared to wild-type Mi_b-CK assayed under the same conditions. This corresponds to a 1.7- and 2.8-fold lower V_{max}, respectively, than the V_{max} of the wild-type enzyme measured under its own optimal conditions. Thus, in the presence of chloride ions, the task of Cys278 in catalysis can be mimicked very well by a small, uncharged residue, with the smallest amino acid substitution performing best.

The determination of V_{max} and K_m(PCr) in the reverse reaction corroborates the observations that have been made with salt and pH titrations. The increased specific activities of the C278G, C278S, and C278N Mi_b-CKs at lower pH values and higher KCl concentration are mostly due to increased catalytic rates (reflected in higher V_{max}) and not to gross changes in substrate saturation under the chosen experimental conditions. The high maximal activities of the mutant enzymes C278G and C278S under optimized conditions underscore the notion that the Cys278 is indeed not essential for catalysis.

Determination of Kinetic Constants for PCr Synthesis (Forward Reaction). For enzymes that bind two substrates, the binding of the first substrate to the free enzyme is described by the constant K_d, and the binding of the second substrate to this complex is described by K_m. For the cytosolic rabbit M-CK isoenzyme, the K_d constants were found to be higher than the K_m constants (Morrison & James, 1965; Maggio et al., 1977). This is characteristic of synergism in substrate binding for a rapid-equilibrium random mechanism (Morrison & Cleland, 1966); i.e., the binding of the second substrate is facilitated by the prior binding of the first. The question of synergistic substrate binding was addressed for Mi_b-CK in the forward reaction at pH 8.0. The data for the wild-type, C278G, and C278S Mi_b-CK enzymes were collected by measuring velocities at four different fixed Cr concentrations with MgATP as the variable substrate. K_m, K_d, and V_{max} values were determined with the fitting programs developed by Cleland (1979). The wild-type Mi_b-CK exhibited synergism for the binding of the second substrate, that is, the K_d values for a given substrate were higher than the corresponding K_m values (Table IV). It was found that the free mutant enzymes C278G and C278S have about a 2.5-fold higher affinity for MgATP and 3- and 5-fold lower affinities for Cr, respectively, than the wild-type enzyme as seen from the corresponding K_d values (Table IV). Interestingly, the two mutants had K_d values for both substrates which were smaller than the corresponding K_m values (Table IV). If we assume

Table III: Michaelis–Menten Constants for ATP Synthesis (Reverse Reaction) at Different pH Values[a]

	pH 5.5		pH 6.0		pH 7.0	
enzyme	K_m(PCr) (mM)	V_{max} (units/mg)[b]	K_m(PCr) (mM)	V_{max} (units/mg)[b]	K_m(PCr) (mM)	V_{max} (units/mg)[b]
wild-type	0.61 ± 0.06	78.2 ± 2.2	0.70 ● 0.05	108.7 ± 1.0	1.30 ● 0.16	88.8 ± 2.2
C278G	7.73 ● 0.54	62.2 ± 4.3	8.28 ● 1.35	43.9 ± 4.5	10.92 ± 0.35	11.3 ± 0.2
C278S	7.85 ± 0.66	39.0 ± 1.9	nd[c]	nd	nd	nd

[a] Reaction conditions were as follows: pH as indicated above the individual columns; 1 mM ADP, 15 mM $MgCl_2$, 65 mM KCl, and 1 mM 2-mercaptoethanol at 25 °C. PCr concentrations were varied in the range of 30 to 0.2 mM for the wild-type enzyme and between 30 and 2 mM for the mutant enzymes. Kinetic parameters presented in Table III are not directly comparable to values presented in Table II (see also comment to Table II). Note that under near-optimal conditions, the maximal activities V_{max} of C278G and C278S mutants are only reduced 1.7-fold and 2.8-fold, respectively, compared to the wild-type enzyme measured under its near-optimal conditions. [b] For unit definition, see Table I. [c] nd, not determined.

Table IV: Kinetic Constants for PCr Synthesis (Forward Reaction)[a]

enzyme	K_d(Cr)[b] (mM)	K_m(Cr) (mM)	K_d(Mg-ATP)[b] (mM)	K_m(Mg-ATP) (mM)	V_{max} (units/mg)[c]
wild-type	19.6 ± 1.6	8.9 ● 0.3	0.70 ± 0.04	0.32 ± 0.02	60.7 ± 0.6
C278G	64 ± 17	273 ± 70	0.27 ± 0.05	1.13 ± 0.37	6.0 ± 1.2
C278S	92 ± 23	209 ● 29	0.31 ± 0.04	0.70 ± 0.16	2.0 ± 0.2

[a] Reaction conditions were 65 mM KCl and 1 mM 2-mercaptoethanol at pH 8.0, 25 °C. Cr concentrations were varied between 7 and 45 mM for the wild-type enzyme and between 40 and 90 mM for the mutant enzymes (four different fixed Cr concentrations each). MgATP concentrations were varied between 0.155 and 8.6 mM for the wild-type enzyme and between 0.155 and 2.33 mM for the mutant enzymes (9–10 MgATP concentrations each). MgATP concentrations higher than 5 mM led to a substrate inhibition-type decrease in reaction velocity for the mutant enzymes (see also text). [Mg^{2+}] was always 1 mM in excess compared to the ATP concentration. [b] For the definition of K_d, see Abbreviations in footnote 1. [c] For unit definition, see Table I.

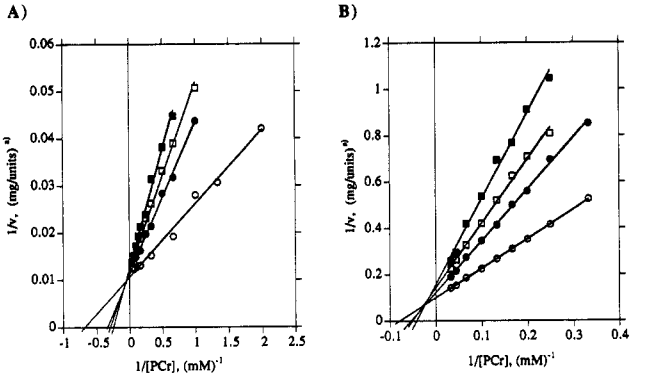

FIGURE 3: Inhibition of wild-type and C278G Mi_b-CKs by free ADP in the reverse reaction. Initial velocities were determined at four different fixed concentrations of free ADP, with PCr as the varying substrate. Reaction conditions were 1 mM 2-mercaptoethanol at pH 7.0 and 25 °C; PCr concentrations were varied from 0.5 to 30 mM for the wild-type and from 3.0 to 30 mM for the C278G Mi_b-CK. The MgADP concentration was kept constant at 1 mM by adjusting total ADP and total Mg^{2+} to obtain the concentrations of free ADP indicated for the individual curves. For the calculation of free ADP concentration, see the Materials and Methods section. (A) Primary Lineweaver–Burk plots for wild-type Mi_b-CK: ○, 0.05 mM free ADP; ●, 2.0 mM free ADP; □, 4.0 mM free ADP; ■, 6.0 mM free ADP. (B) Primary Lineweaver–Burk plots for C278G Mi_b-CK: ○, 0.05 mM free ADP, ●, 0.2 mM free ADP; □, 0.3 mM free ADP; ■, 0.4 mM free ADP. For the determination of the competitive inhibition constant K_I and K_m(real) for PCr, see Table V. [a]For unit definition, see Table I. Note the significantly higher inhibition of C278G mutant by free ADP compared to wild-type enzyme.

Table V: Inhibition Constants (K_I) of Free ADP with Respect to PCr in the Reverse Reaction[a]

enzyme	K_I(competitive) (mM)	K_m(real)(PCr) (mM)	V_{max} (units/mg)[b]
wild-type	1.96 ± 0.07	1.29 ● 0.04	89.1 ± 0.6
C278G	0.090 ± 0.005	7.23 ● 0.39	9.4 ± 0.2

[a] Competitive inhibition constants K_I and the real Michaelis–Menten constants were determined from initial velocities at four different fixed free ADP concentrations (0.05, 2.0, 4.0, and 6.0 mM for the wild-type Mi_b-CK; 0.05, 0.2, 0.3, and 0.4 mM for C278G Mi_b-CK) with PCr as the variable substrate and a constant concentration of 1 mM MgADP (for the primary data, see Figure 3A,B). Mg^{2+} was added as $MgAc_2$; assay mixtures also contained 65 mM KCl and 1 mM 2-mercaptoethanol at pH 7.0 and 25 °C. The real Michaelis–Menten constants are in good agreement with the constants shown in Table III, which were determined under low free ADP conditions. [b] For unit definition, see Table I.

that the mutants still follow the same reaction mechanism as the wild-type enzyme, this new relationship between K_d and K_m values indicates that both mutant enzymes have lost the property of synergistic substrate binding. For these two altered enzymes, the two substrates act like competitive inhibitors with respect to each other, since the substrate first bound hinders the binding of the second substrate.

Inhibition by Nucleotides. While measuring enzyme activities for the reverse reaction, we observed that the activities of all mutant Mi_b-CKs measured at 4 mM ADP and 8.5 mM $MgCl_2$ were lower than their activities at 1 mM ADP and 8.5 mM $MgCl_2$. In contrast, the wild-type enzyme showed similar activities under each of these conditions. To quantitate the observed effect, the inhibition constants of free ADP as inhibitor with respect to PCr were determined for the wild-type and C278G Mi_b-CKs at pH 7.0. The observed inhibition was of the mixed type for the wild-type and for the C278G enzyme (i.e., the lines in the primary Lineweaver–Burk plot did not intersect exactly on the $1/v$ axis; Figure 3A,B); that is, it had a competitive and a noncompetitive component. Similar, but not completely conclusive, findings were reported earlier (Morrison & O'Sullivan, 1965). The competitive inhibition component was clearly predominant over the noncompetitive. The competitive inhibition constant K_I for free ADP for the C278G Mi_b-CK is about 22-fold lower than for the wild-type enzyme and explains the high sensitivity of the mutant enzymes toward inhibition by free ADP (Table V).

In the forward reaction, the mutant enzymes C278G and C278S showed a slight decrease in activity at MgATP concentrations above 5 mM. The shape of the curve (velocity vs MgATP concentration) was very reminiscent of that of substrate inhibition (not shown). Since commercial ATP sources always contain small amounts of contaminating ADP (<1%), it was determined whether the mutant enzyme C278G was more prone to inhibition by MgADP than the wild-type enzyme. For both the wild-type and mutant enzymes, the inhibition by MgADP was purely competitive with respect to MgATP and noncompetitive with respect to creatine (not shown). However, the mutant C278G Mi_b-CK was more sensitive to inhibition by free ATP than the wild-type enzyme. The question of whether this increased sensitivity to ATP inhibition was responsible for the "substrate-type" inhibition of the mutant enzymes C278G and C278S cannot be clearly answered. The binding constant for Mg^{2+} with ATP seems

to be high enough to ensure an almost complete absence of free ATP in solutions when Mg^{2+} is in a 1 mM excess.

Although the changes in the inhibition pattern between the wild-type and mutant Mi_b-CKs were not fully elucidated and quantitated, the increased sensitivity of the mutant enzymes to inhibition by free nucleotides must be taken into account. Concentrations of free ADP as they are found in some assay mixtures can already cause substantial inhibition, thereby giving rise to altered apparent kinetic constants and to an artificially low estimate of the performance of enzymes with substitutions at the reactive cysteine. Measurements at high MgATP concentrations (>5 mM) can also lead to lower estimates of the activity of the mutant enzymes than measurements taken at low MgATP concentrations.

DISCUSSION

Phosphagen kinases are efficiently inactivated by thiol-modifying reagents. For the creatine kinases (CK) it has been shown that the conserved cysteine residue at position 282 (homologous to cysteine residue 278 in the mitochondrial isoenzymes) is highly reactive. It is still a matter of dispute whether or not the reactive cysteine is essential for CK activity. With many of the modifying reagents, modification of one cysteine per protomer led to complete loss of enzymatic activity, suggesting that this cysteine is essential [Mahowald et al., 1962; reviewed in Kenyon and Reed (1983)]. However, other studies have concluded that the reactive cysteine is not essential for CK activity since modification with some small, uncharged groups caused only partial inactivation (Smith & Kenyon, 1974; der Terrossian & Kassab, 1976) with concomitant loss of synergistic substrate binding (Maggio et al., 1977). These latter studies have been challenged in more recent investigations (Reddy & Watts, 1979; Wu et al., 1989; Zhou & Tsou, 1987), in which it was pointed out that chemical modifications might not yield a completely homogeneous enzyme population due to incomplete reaction or due to intramolecular transfer of the thiol-modifying group which would restore an active enzyme. Use of site-directed amino acid substitutions at the position of the reactive cysteine avoids the problems inherent with chemically modified CK. We substituted the reactive cysteine in the mitochondrial creatine kinase isoenzyme (Mi_b-CK) by glycine, serine, alanine, asparagine, and aspartate and then purified the mutant proteins from overexpressing *E. coli* strains. When assayed under standard conditions, these mutant enzymes still showed low residual activity, but in the absence of chloride ions, the activities of the mutant enzymes were minimal (Table I). However, the maximal activities (V_{max}) of C278G and C278S, if measured under near-optimal conditions, were reduced only 1.7- and 2.8-fold, respectively, compared to maximal wild-type Mi_b-CK activities (Table III). This discrepancy emphasizes the need to optimize assay conditions for new mutant forms of an enzyme in order to correctly evaluate the effect of the amino acid substitution on the performance of the protein. Viewing the activities of the mutant enzymes under all tested conditions, we conclude that the reactive cysteine is nonessential for CK activity, as has also been postulated previously (Smith & Kenyon, 1974; Smith et al., 1975; der Terrossian & Kassab, 1976).

Nevertheless, this study has shown that the negative charge provided by the cysteine residue is important for maximal function of the enzyme *in vitro*. At very low Cl^- concentrations, substitution of cysteine with the charged aspartate allowed a 12-fold higher V_{max} than replacement by its uncharged counterpart asparagine (Table II). The activities of all mutant Mi_b-CKs with uncharged substitutions were increased severalfold by Cl^- and Br^- ions (Tables I and II; Figure 2). Thus, a negative charge to increase the activity of mutant Mi_b-CK can also be provided in the form of Cl^- ions and not only through a negatively charged side chain. The activation by Cl^- occurs at the level of V_{max} and not K_m values. Altered substrate binding is probably not the main determinant for increased activity, as K_m values are actually increased with increasing Cl^- concentrations (Table II). Spatial constaints are clearly a factor in this activation, since a large number of other anions did not activate and since the larger Br^- ions did not activate as well. The size of the substituted amino acid is also important, with small uncharged substitutions being activated to a higher extent than larger substitutions (Tables I and II; Figure 2). These spatial constraints suggest that Cl^- exerts its effect in the active site, acting on the catalytic step. Activation could be due to either stabilization of a transition-state complex, enhancement of the transphosphorylation step, or facilitation of product release. Stabilization of the transition-state complex is unlikely, since other anions which stabilize the transition-state complex (e.g., NO_2^- and NO_3^-, which like Cl^- are also members of the "group two" anions; Milner-White & Watts, 1971) do not activate the mutant Mi_b-CKs at all.

The importance of a negative charge for high enzyme activity of the mutant Mi_b-CKs suggests that the cysteine side chain in the wild-type enzyme is in its deprotonated state for maximal activity. This is in agreement with earlier studies which showed pH-independent modification/inactivation of CK by iodoacetamide between pH 6.0 and 9.5. It was also shown that modification of CK with iodoacetate preserves the overall charge while iodoacetamide reduced the charge by one unit per protomer [reviewed in Watts (1973)].

It has been proposed that Cys282 (cytosolic CKs), in its deprotonated state, plays an integral part in a proton flow circuit to facilitate the phosphoryl transfer [reviewed in Watts (1973)]. In this and in alternative models, a histidine residue is also proposed to be involved in catalytic proton exchange (Cook et al., 1981; Rosevear et al., 1981). Our data do not support a role for the reactive cysteine in such a proton flow circuit, since substitution of the reactive cysteine with amino acids that interrupt the proton flow would be expected to lead to an increase in the pK_a of the catalytic histidine. Rather than the predicted shift of pH optima to higher pH values, a shift of pH optima toward lower pH values was observed for all of the mutants investigated (Figure 1; Table III). This shift toward lower pH values is still consistent with the presence of a catalytic histidine, since cysteine substitutions could induce a conformational change in the active site which then could indirectly change the environment of the histidine, causing a pK_a shift of the histidine residue to a lower value.

The role of the reactive cysteine in substrate binding could be analyzed by the determination of the K_d and K_m values for the forward reaction (PCr synthesis). In mutants C278G and C278S, K_m and K_d values for both substrates were affected. The free enzyme containing either of these amino acid substitutions had about a 2.5-fold higher affinity for MgATP and 3- and 5-fold lower affinities for creatine, respectively, than the wild-type enzyme (Table IV). If the cysteine residue were to participate directly in the binding of creatine, we would expect more dramatically increased K_d(Cr) values for the mutant enzymes, and as the K_d(MgATP) values are even lower for the mutant enzymes, a specific and direct interaction between MgATP and Cys278 can almost be ruled out. However, the observed differences in K_d argue that the cysteine

may be *indirectly* involved in the binding of the two substrates MgATP and creatine. Hence, these results validate the earlier studies that favored a role for this cysteine in ATP binding (Maggio et al., 1977; Markham et al., 1977) and also those which supported a role in creatine binding (Buechter et al., 1992).

Wild-type Mi_b-CK showed synergistic substrate binding (Table IV). It has been postulated that conformational changes induced by the binding of the first substrate to the free wild-type enzyme facilitate the binding of the second substrate. For C278G and C278S, however, the K_d values were smaller than the corresponding K_m values (Table IV). If we assume that the mutants follow the same reaction mechanism as the wild-type enzyme, they do not show synergistic binding but rather display the opposite behavior; i.e., the binding of the first substrate makes binding of the second substrate more difficult. This implies that, in the absence of Cys278, no or incorrect conformational changes occur upon binding of the first substrate. Thus, the altered K_d and K_m values for C278S and C278G show that Cys278 is necessary for synergistic substrate binding and suggest a conformational role of the reactive cysteine in shaping the correct spatial arrangement of the two substrate binding sites. This cysteine might function as a hinge to transmit conformational changes upon binding of the first substrate. Similar conclusions have been presented earlier, based on partial loss of synergism of chemically modified rabbit muscle CK in conjunction with a secondary structure prediction in the vicinity of Cys282 (Maggio et al., 1977). This prediction placed Cys282 at the beginning of a β-turn which separates two portions of β-sheet structure.

Comparison of the K_d(MgATP) of C278G and C278S to the K_m(MgATP) of the wild-type enzyme (Table IV) suggests that the affinity of the free mutant enzymes for MgATP is similar to the "enhanced" affinity of wild-type Mi_b-CK complexed with creatine for MgATP. One interpretation of this result is that the mutants have a "constitutive" conformational change at the active site that facilitates the binding of MgATP, but not of creatine, to the free mutant enzyme. It is also possible that MgATP binds better to the free mutant enzyme simply due to the removal of the negative charge of the thiolate.

The conservative substitution of serine for cysteine does not allow maximum activity or synergistic binding, despite the fact that the serine side chain is most like the cysteine side chain in length. In fact, the properties of C278S are similar to those of C278G, even though glycine is the smallest substitution possible. The failure of serine to substitute distinctly better than glycine implies that the unique ability of cysteine to form hydrogen bonds, salt bridges, or even temporary covalent linkages is important. These properties would be well suited for the dual role of the cysteine residue which we propose. Its negative charge is important for the maximal activity of the creatine kinase, but a direct role in catalysis is not supported by our data. While the undramatically altered K_d values in the C278S and C278G Mi_b-CKs argue for an indirect role in the binding of MgATP and creatine, the cysteine is necessary for synergistic substrate binding, suggesting that it is involved in shaping and maintaining the conformation between the substrate binding sites. The exact role of cysteine can be further clarified with a high-resolution structure in hand, in combination with more substitutions of other amino acids forming the active site.

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REFERENCES

Borders, C. L., & Riordan, J. F. (1975) *Biochemistry 14*, 4699–4704.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.
Buechter, D. D., Medzihradszky, K. F., Burlingame, A. L., & Kenyon, G. L. (1992) *J. Biol. Chem. 267*, 2173–2178.
Cleland, W. W. (1979) *Methods Enzymol. 63*, 103–138.
Cook, P. F., Kenyon, G. L., & Cleland, W. W. (1981) *Biochemistry 20*, 1204–1210.
der Terrossian, E., & Kassab, R. (1976) *Eur. J. Biochem. 70*, 623–628.
Eppenberger, H. M., Eppenberger, M., Richterich, I., & Aebi, H. (1964) *Dev. Biol. 10*, 1–16.
Fedosov, S. N., & Belousova, L. V. (1988) *Biochemistry USSR 53*, 478–491.
Furter, R., Kaldis, P., Furter-Graves, E. M., Schnyder, T., Eppenberger, H. M., & Wallimann, T. (1992) *Biochem. J. 288*, 771–775.
Jacobs, H., Heldt, H. W., & Klingenberg, M. (1964) *Biochem. Biophys. Res. Commun. 16*, 516–521.
Jacobus, W. E., & Lehninger, A. L. (1973) *J. Biol. Chem. 248*, 4803–4810.
James, T. L. (1976) *Biochemistry 15*, 4724–4730.
James, T. L., & Cohn, M. (1974) *J. Biol. Chem. 249*, 2599–2604.
James, P., Wyss, M., Lutsenko, S., Wallimann, T., & Carafoli, E. (1990) *FEBS Lett. 273*, 139–143.
Kassab, R., Roustan, C., & Pradel, L.-A. (1968) *Biochem. Biophys. Acta 167*, 308–316.
Kenyon, G. L., & Reed, G. H. (1983) *Adv. Enzymol.* (Meister, A., Ed.) *54*, 367–426.
Maggio, E. T., Kenyon, G. L., Markham, G. D., & Reed, G. H. (1977) *J. Biol. Chem. 252*, 1202–1207.
Mahowald, T. A., Noltmann, E. A., & Kuby, S. A. (1962) *J. Biol. Chem. 237*, 1535–1548.
Markham, G. D., Reed, G. H., Maggio, E. T., & Kenyon, G. L. (1977) *J. Biol. Chem. 252*, 1197–1201.
Milner-White, E. J., & Watts, D. C. (1971) *Biochem. J. 122*, 727–740.
Morrison, J. F., & James, E. (1965) *Biochem. J. 97*, 37–52.
Morrison, J. F., & O'Sullivan, W. J. (1965) *Biochem. J. 94*, 221–235.
Morrison, J. F., & Cleland, W. W. (1966) *J. Biol. Chem. 241*, 673–683.
Nakamaye, K. L., & Eckstein, F. (1986) *Nucleic Acids Res. 14*, 9679–9698.
O'Sullivan, W. J., & Smithers, G. W. (1979) *Methods Enzymol. 63*, 294–336.
Reddy, S. R. R., & Watts, D. C. (1979) *Biochim. Biophys. Acta 569*, 109–113.
Rosevear, P. R., Desmeules, P., Kenyon, G. L., & Mildvan, A. S. (1981) *Biochemistry 20*, 6155–6164.
Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Schlegel, J., Wyss, M., Eppenberger, H. M., & Wallimann, T. (1988) *J. Biol. Chem. 263*, 16963–16969.
Smith, D. J., & Kenyon, G. L. (1974) *J. Biol. Chem. 249*, 3317–3318.
Smith, D. J., Maggio, E. T., & Kenyon, G. L. (1975) *Biochemistry 14*, 766–771.

Studier, F. W., Rosenberg, A. H., Dunn, J. J., & Dubendorff, J. W. (1990) *Methods Enzymol. 185*, 60–89.
Vasák, M., Nagayama, K., Wüthrich, K., Mertens, M. L., & Kägi, J. H. R. (1979) *Biochemistry 18*, 5050–5055.
Wallimann, T. Schlösser, T., & Eppenberger, H. M. (1984) *J. Biol. Chem. 259*, 5238–5246.
Wallimann, T., Wyss, M., Brdiczka, D., Nicolay, K., & Eppenberger, H. M. (1992) *Biochem. J. 281*, 21–40.
Watts, D. C. (1973) *The Enzymes*, 3rd ed., Vol. 8, pp 383–455, Academic Press, New York.
Wu, H., Yao, Q.-Z., & Tsou, C.-L. (1989) *Biochim. Biophys. Acta 997*, 78–82.
Wyss, M. (1992) Ph.D. Thesis, ETH, Zürich, Switzerland, No. 9777.
Wyss, M., Smeitink, J., Wevers, R. A., & Wallimann, T. (1992) *Biochim. Biophys. Acta 1102*, 119–166.
Wyss, M., Wallimann, T., & Köhrle, J. (1993) *Biochem. J. 291*, 463–472.
Zhou, H.-M., & Tsou, C.-L. (1987) *Biochim. Biophys. Acta 991*, 136–143.