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Mutation of *cis*-proline 207 in mitochondrial creatine kinase to alanine leads to increased acid stability

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We show that the mutation of an uncharged residue far from the active site to another uncharged residue can have effects on the active site without disturbing the overall structure of the protein. *Cis*-proline 207 of mitochondrial creatine kinase was mutated to alanine. The mutant showed a decrease in the pH-optimum for ATP synthesis by 1.5 units while the maximum relative activity was lowered to 53% of the wild-type enzyme. In the direction of ATP consumption, the pH optimum was lowered by 1.3 units and the maximum relative activity was 49% of the wild-type enzyme. The enzyme kinetic parameters K_m and K_d for the substrates did not change dramatically, indicating a largely unperturbed active site. Small-angle X-ray scattering was used to investigate the structural change concomitant with the mutation, yielding a scattering profile only slightly different from that of the wild-type enzyme. Neither the radius of gyration nor the molecular mass showed any significant differences, leading to the conclusion that quarternary organization and fold of the mutant and the wild-type enzymes were similar. Theoretical analysis suggests the most probable primary source of structural change to be a transition of residue 207 peptide bond torsional angle ω from the *cis* to the *trans* configuration.

Keywords: cis-proline/creatine kinase/site-directed mutagenesis/small-angle scattering/mitochondrial protein

Introduction

Creatine kinase (CK; EC 2.7.2.3) is a key enzyme of cellular energy metabolism, catalyzing the reversible phosphoryl transfer from phosphocreatine (PCr) to ADP yielding creatine (Cr) and ATP (for a review see Wallimann *et al.*, 1992). Creatine kinase is present in vertebrate cells of high and fluctuating energy demand, e.g. muscle fibres, neurons, photoreceptors, spermatozoa or electrocytes, where it regenerates ATP from phosphocreatine during cellular work. Several isoforms of the enzyme have been reported, cytosolic (M, muscle type; B, brain type) and mitochondrial isoenzymes. Three cytosolic isoenzymes exist in homo- or heterodimeric form (BB-, MB- and MM-CK) and two mitochondrial CKs occur mainly as octamers (Mi_a- and Mi_b-CK) (Wyss *et al.*, 1992). The octameric assemblies of Mi-CK are exclusively formed by the assembly of dimeric building blocks that seem to have exceptional stability against disruption into monomers (Gross and Wallimann, 1993). No active monomeric creatine kinase has been described so far, neither as a wild type enzyme nor in mutational studies.

Chicken muscle Mi_b-CK has been crystallized in our laboratory (Schnyder *et al.*, 1990) and its X-ray structure in the presence of bound ATP has been solved (Fritz-Wolf *et al.*, 1996). Analysis of the peptide bond torsional angles revealed that one of the 22 proline residues of Mi_b-CK is in *cis* conformation. This residue, proline 207 is conserved throughout the family of guanidino kinases (Mühlebach *et al.*, 1994) and is positioned in a loop that is involved in monomer–monomer contacts within the dimeric building blocks of the enzyme. Upon mutation of the neighbouring residue Trp206, no functional octameric Mi_b-CK assemblies are formed under conditions where the wild-type enzyme is in its fully octameric state (Gross *et al.*, 1994), demonstrating the importance of this region for the structural and functional integrity of creatine kinase. In Figure 1 we show the monomer–monomer contact region within a dimeric building block of Mi_b-CK as determined by X-ray crystallography (Fritz-Wolf *et al.*, 1996).

Cis–trans isomerization of prolines has been shown to play an important role in the folding process of enzymes (Brandts *et al.*, 1975). So far, no information exists about the special role of *cis*-prolines in proteins, except that they provide a special orientation of the peptide bond. We have been studying the unfolding process of guanidino kinases by different biophysical methods and found that mutation of Trp206 hindered proper folding of the enzyme (Gross *et al.*, 1994). By mutation of the vicinal residue Pro207, we expected to change the conformation of the monomer–monomer interface in a similar way and prevent the assembly of dimers from the monomeric building blocks or even the correct folding of the monomers themselves.

In order to investigate the function of the *cis*-proline 207, we have exchanged the residue against alanine by site-directed mutagenesis and used small-angle X-ray scattering to study the effects of the mutation on the enzyme's overall structure.

Materials and methods

Inverse PCR (IPCR)

pRF23, coding for the wild-type (wt) Mi_b-CK (Furter *et al.*, 1992) was used as a template in all IPCR experiments. In order to amplify whole plasmids as templates while avoiding random mutations, DNA polymerase from *Pyrococcus furiosus* (Stratagene) was used. The reactions were initiated by adding the enzyme to the denatured DNA template at 95°C. Routinely 25 cycles of PCR were performed (95°C, 1 min; 52°C, 1 min; 72°C, 7 min), followed by a 30 min final extension step at 72°C. The following primers were used: Mi620H (5′-GCGGATGCCAGAGGAATCT-3′), Mi619Z (5′-CCAGTCACGAGCCATCCCA-3′) corresponding to nucleotides 620–638 and 619–601 of the coding sequence for Mi_b-CK, respectively. The linear PCR products were circularized using T4 DNA ligase, followed by transformation into *Escherichia coli* XL1-

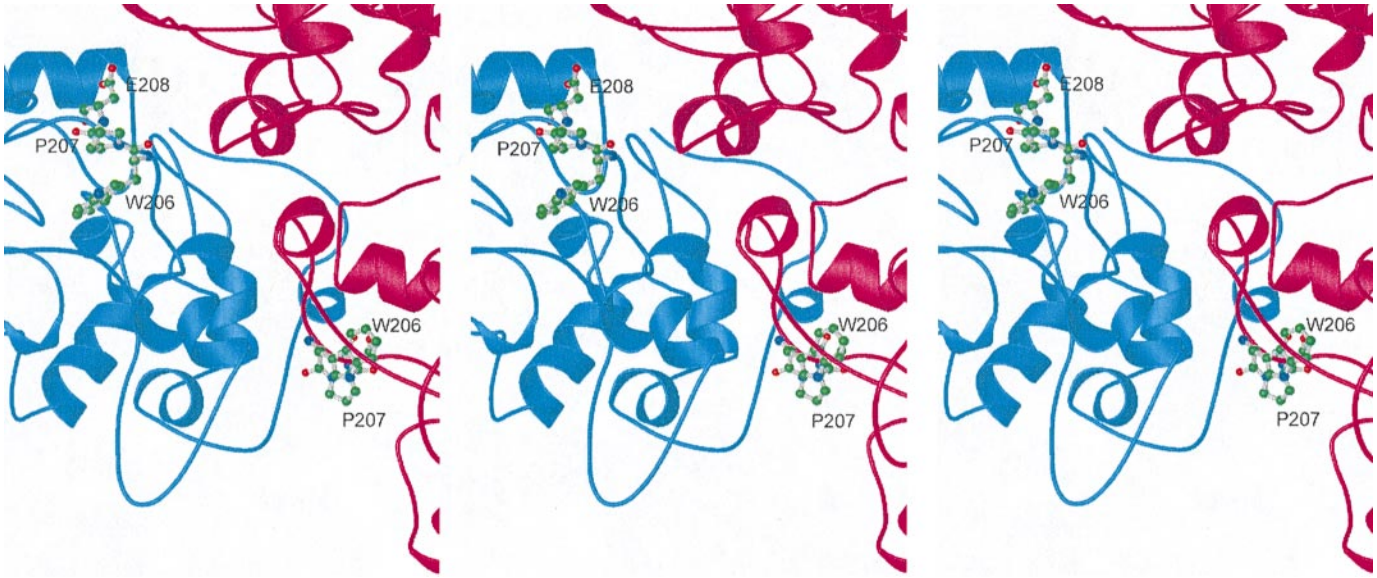

Fig. 1. The structure of the monomer–monomer interface region of Mi_b-CK. A stereo view of the monomer–monomer interface region from one dimeric building block of octameric Mi_b-CK is shown. The monomers are colored in magenta and cyan, respectively, with residues Pro207 and the neighbouring residues Trp206 and Glu208 drawn in ball-and-stick mode. Note the 'stacking' orientation of Pro207 and the aromatic ring of residue Trp206. The figure was prepared from the coordinates of Mi_b-CK (Fritz-Wolf *et al.*, 1996) using Molscript (Kraulis, 1991) and rendered using Raster3D V2.3 (Merritt and Bacon, 1997).

Blue competent cells and plasmids were prepared and analyzed following routine procedures (Ausubel *et al.*, 1992). All mutated plasmids were checked for the success of *in vitro* mutagenesis by sequence analysis using the dideoxy chain termination method (Sanger *et al.*, 1977; Tabor and Richardson, 1987).

Protein purification

Escherichia coli cells of strain BL-21(DE3)lysS were transformed with plasmids coding for the mutant and wild-type enzymes and proteins were expressed and purified as described previously (Furter *et al.*, 1992). All protein preparations were homogeneous as judged by Coomassie blue-stained SDS-polyacrylamide gels which were also used to monitor the purification steps. Protein concentrations were determined by the BioRad method (Bradford, 1976) using BSA as a standard.

Measurement of enzyme activity

Enzyme activity was measured by the pH-stat method (Milner-White and Watts, 1971; Wallimann *et al.*, 1984) in a buffer containing 75 mM KCl, 10 mM $MgCl_2$, 0.1 mM EGTA [ethyleneglycol-bis(2-aminoethylether)tetraacetic acid] and 1 mM β-ME (2-mercaptoethanol). For routine checks of enzyme activity, phosphocreatine and ADP were added to a final concentration of 10 mM each and the reaction was performed at pH 7.00. The pH-dependence of the reaction for all enzymes was checked in steps of 0.25 pH units in the areas of the peaks and 0.5 pH units elsewhere. For performing enzyme kinetics studies in the forward reaction (ATP + Cr → ADP + PCr + H^+), the reaction conditions were 75 mM KCl, 0.1 mM EGTA, 16 mM $MgAc_2$ and 1 mM β-ME at pH 8.0, 25°C. Creatine concentrations were varied between 7 and 45 mM for the wild-type enzyme and between 20 and 70 mM for the mutant enzyme. ATP concentrations were varied between 0.155 and 2.33 mM for the wild-type and mutant enzymes. In the reverse reaction (ADP + PCr + H^+ → ATP + Cr), the assay mixture for the wild-type and the mutants contained 75 mM KCl, 10 mM $MgCl_2$, 0.1 mM EGTA and 1 mM β-ME at pH 7.0, 25°C. For the determination of PCr K_m values, the concentration of ADP was 4 mM. Phosphocreatine concentrations were varied between 30 and 0.2 mM for wild type and 30 and 1 mM for mutant enzymes. For the determination of ADP K_m values, the concentration of PCr was 10 mM and the ADP concentration was varied between 1 and 0.1 mM for wild-type and mutant enzymes. The evaluation of the kinetic parameters was performed with a computer program by W.W.Cleland (1979), adapted for personal computer by R.Viola (Akron University, Akron, OH). Standard errors are given for the calculated values; each set of constants represents the mean of three or four independent measurements. 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, and E represents the enzyme. V_{max} denotes the maximal initial velocity as determined from Michaelis–Menten kinetics.

Small-angle X-ray scattering

Small-angle X-ray scattering (SAXS) measurements were carried out using a modified Kratky compact camera (MBraun-Graz-Optical Systems, Graz, Austria; Laggner and Mio, 1992), equipped with a thermostatically controlled sample holder at 10 or 20°C using a quartz capillary with a volume of 60 µl and an internal diameter of 1.0 mm. Protein concentration series of 2.5, 5, 7.5, 10, 12.5 and 15 mg/ml were used. The primary X-ray beam of 1.5418 Å wavelength (Cu-Kα radiation) was produced by a water cooled rotating anode generator (Rigaku Corp., Japan) and Ni-filtering. The sample-to-detector distance was 272 mm and the position calibration of the detector was performed using Ag-stearate as a reference material. The scattering curve was monitored in an s-range between 0.05 and 0.2 nm^{-1} ($s = 2 \sin(\theta/\lambda)$, with 2θ being the scattering angle and $\lambda = 0.15418$ nm being the wavelength of the X-rays). Exposure times were typically 1000 s for individual measure-

ments. For data analysis, the results of three individual measurements were merged, background subtraction was performed and the resulting data were deconvoluted with the slit width and slit length profiles of the primary beam and subsequently subjected to Fourier transformation using the program ITP (Glatter, 1977). Radii of gyration were determined by the Guinier approximation from the low-s regions of the scattering curves employing the PS software (MBraun-Graz-Optical Systems, Graz, Austria) supplied with the SAXS detector, yielding also the intensity extrapolated to zero angle that was used in the subsequent calculation of relative molecular masses according to Luzatti (1960). To rule out radiation damage, enzymatic activities were determined from samples recovered from SAXS experiments.

Results

Mutagenesis, purification, activity and kinetic analysis

The amino acid residue proline 207 of chicken Mi_b-CK was mutated to yield the alanine mutant and the mutated protein was expressed in *E.coli* as a soluble protein under the control of the T7 promoter (Studier *et al.*, 1990) and purified as described (Furter *et al.*, 1992). On average, 20 mg pure protein could be isolated from one liter of *E.coli* culture, compared with average yields of 50–60 mg/l for the wild-type enzyme. The activities of the mutant enzyme as a function of the pH were measured in both forward (ATP consumption) and reverse (ATP synthesis) reaction directions and compared with the wild-type enzyme. Figure 2 shows the pH dependent activity of Pro207Ala compared with the wild-type. In the reverse reaction, a shift of the pH optimum by 1.5 units towards the acidic side resulted from the mutation, lowering the pH optimum to 4.5. This shift was concomitant with a general change of the curve shape (Figure 2B). The relative activity at the pH optimum was decreased to 53% of the wild-type activity at its respective pH optimum of 6.0. In the direction of ATP consumption (forward reaction), a similar decrease of the pH optimum by 1.3 units was observed, with a significant narrowing of the pH area optimal for the reaction (Figure 2A). The mutant's maximal activity was decreased to 49% that of wild type.

No significant changes in K_m or K_d for the substrates ATP and creatine were observed. In the reverse reaction, K_m values for both substrates remained largely unaffected. V_{max} values were lowered by approximately 1/3 in both directions of the reaction coordinate. The kinetic parameters for the substrates ATP, ADP, Cr and PCr are shown in comparison to the wild type enzyme in Tables I and II.

Small-angle X-ray scattering

Small-angle X-ray scattering showed a small change in the radius of gyration (R_g) from 55.6 ± 0.9 Å for the wild-type enzyme to 56.8 ± 1.0 Å for Pro207Ala as determined from the Guinier representations of the low-angle parts of the respective scattering curves (see also Forstner *et al.*, 1996 and Forstner *et al.*, submitted). The difference between these values was determined by a two-tailed statistical test not to be significant at any practical level. We determined the relative molecular masses (M_r) of the proteins from the scattering profiles according to the relationship of M_r to the normalized intensity extrapolated to zero-angle (Luzatti, 1960), yielding a value of 340 ± 10 kDa for Pro207Ala, in good agreement with a value of 344 kDa calculated for the octameric enzyme. This relation showed no dependence on protein concentration in our

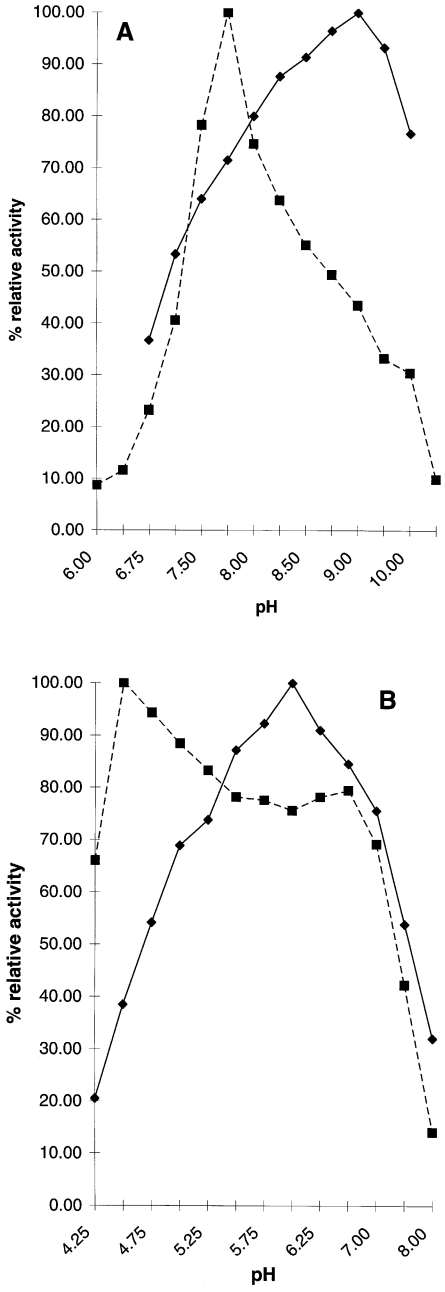

Fig. 2. pH optimum curves for wild type and mutant Mib-CK enzymes. Relative activities (ratio of initial rates) as a function of the reaction pH are shown. For comparison the maximum activity of each enzyme was set at a nominal value of 100%. The absolute specific activity of the mutant, however, is only 49% that of wild type in the forward and 53% in the reverse direction of the reaction. (**A**) Forward reaction (ATP consumption). Reaction conditions were 45 mM Cr, 5.4 mM ATP, 75 mM KCl, 0.1 mM EGTA, 16 mM $MgAc_2$ and 1 mM β-mercaptoethanol at 25°C. (**B**) Reverse reaction (ATP synthesis). Reaction conditions were 10 mM PCr, 1 mM ADP, 75 mM KCl, 10 mM $MgCl_2$, 0.1 mM EGTA and 1 mM β-mercaptoethanol at 25°C. Wild type, solid line, ◆; mutant: dashed line, ■).

Table I. Enzyme kinetic parameters for Pro207Ala and wild-type Mi_b-CK/ forward reaction

Enzyme	K_m(ATP) (mM)	K_d(ATP) (mM)	K_m(Cr) (mM)	K_d(Cr) (mM)	V_{max} (U/mg)
Wild type	0.31 ± 0.02	0.72 ± 0.04	8.80 ± 0.31	20.4 ± 3.2	61.7 ± 0.82
Pro207Ala	0.33 ± 0.08	0.71 ± 0.05	11.9 ± 0.77	25.4 ± 4.0	48.5 ± 0.67

Table II. Enzyme kinetic parameters for Pro207Ala and wild-type Mi_b-CK/ reverse reaction

Enzyme	K_m(ADP) (mM)	K_m(PCr) (mM)	V_{max} (U/mg)
Wild type	0.17 ± 0.04	1.21 ± 0.11	92.2 ± 1.0
Pro207Ala	0.17 ± 0.051	1.86 ± 0.16	73.4 ± 3.9

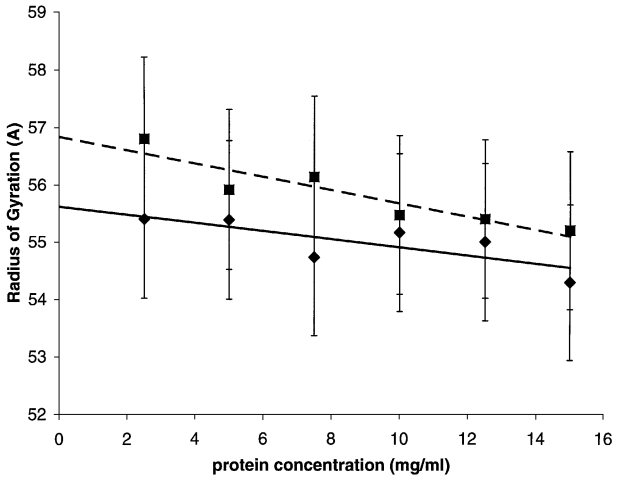

Fig. 3. Concentration-dependence of the radii of gyration. Samples of wild-type and mutant Mi_b-CK were measured at concentrations of 2.5, 5, 7.5, 10, 12.5 and 15 mg/ml at 10 or 20°C, respectively. The radii of gyration determined by the Guinier approximation from the low-angle regions of the scattering curves are plotted against the protein concentration. Extrapolation to infinite dilution yields the R_g values for the wild-type and mutant proteins. Wild-type: solid line, ◆; mutant: dashed line, ■).

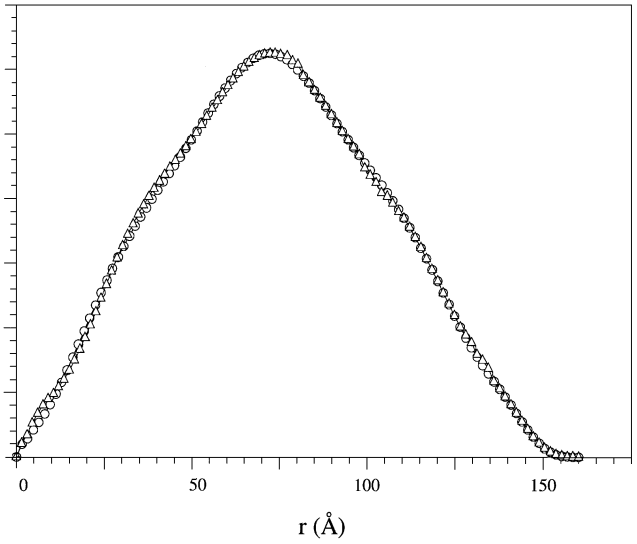

Fig. 4. Distance distribution curves from small-angle X-ray scattering. The P(r) functions were calculated from the small-angle X-ray scattering curves of wild type and mutant Mi_b-CK enzymes using the program ITP (Glatter, 1977). Both enzymes were investigated in their 'open', substrate-free conformation (Forstner *et al.*, 1996). Wild type, circles; mutant, triangles.

experiments (Figure 3) indicating that no protein aggregation occurred as a function of protein concentration. To rule out radiation damage of the samples during SAXS experiments, protein samples were recovered from the cuvettes used in the scattering experiments and analyzed for enzymatic activity. No significant decrease of enzymatic activity was observed.

After desmearing of the scattering curves and background reduction, the radially averaged Fourier transforms of the scattering curves were calculated using the program ITP (Glatter, 1977) to yield the distance distribution functions P(r) and the desmeared radii of gyration of both wild-type and mutant Mi_b-CKs. The compared P(r) curves are shown in Figure 4. The R_g values were 55.2 ± 0.8 Å (wild-type Mi_b-CK) and 56.3 ± 0.5 Å (mutant), insignificantly different from the values determined by Guinier approximation.

Discussion

We have successfully mutated proline residue 207 of chicken Mi_b-CK to an alanine residue, expressed the mutant protein and purified it to homogeneity. The substrate binding site of Mi_b-CK remains largely unaffected by the mutation as demonstrated by the absence of large changes in the parameters describing the binding ability of the substrates, K_m and K_d (Tables I and II). Creatine kinase has been known to exhibit synergism of substrate binding for the substrates creatine and ATP (Maggio *et al.*, 1977). Several mutations, e.g. the mutation of the reactive cysteine C278 (Furter *et al.*, 1993) or the active site histidines (Forstner *et al.*, 1997) have been demonstrated to lead to a loss of the synergistic effect. As the relation between K_m and K_d remains unchanged in mutant Pro207Ala, we conclude that synergism of substrate binding is not influenced by the mutation, giving further evidence that the active site structure itself was indeed conserved.

A large change in the pH optima for both the reverse and forward directions of the creatine kinase reaction was observed with the Pro207Ala mutant, showing decreases of 1.5 and 1 pH units for the reverse and forward reaction, respectively. As the mutated amino acid residue is at a distance more than 15 Å away from the active site, an explanation for this observation is not obvious. We assume, however, that the mutation leads to subtle changes in the structure of the enzyme which in turn lead to a greater exposure of negatively charged side chains. These could subsequently result in a change in surface charge analogous to the change of ionic strength in nonenzymatic ionic reactions or to (primary or secondary) salt effects in physical organic chemistry. The observed shift of the pH optimum of enzymatic activity towards the acidic side would be in agreement with a negatively charged surface of the enzyme destabilizing the supposed basic active site structure. We do not know yet, however, which amino acid(s) of which pK_a(s) in the active site of creatine kinase cause(s) the pH dependence of the reaction. Similar considerations have been made for chymotrypsin, showing that perturbation of the pK_a of a buried group is a complex function of the size and the shape of the protein and also of the ionic strength of the solution (Matthews *et al.*, 1981; Voet *et al.*, 1981).

To further investigate possible structural changes that lead to the observed effects on the pH optima of the creatine kinase reactions in both directions we have studied the low resolution structures of wild-type and mutant Mi_b-CK by SAXS, which is a very useful method for the detection of changes in a protein structure in the absence of a detailed crystal structure (Svergun and Feigin, 1987). The directly determinable parameters R_g and M_r allowed us to draw conclusions on the overall shape and size of the Mi-CK molecule investigated. In our studies we found no significant changes of these parameters upon mutation compared with the data for the wild-type enzyme (Forstner

et al., 1996 and Forstner *et al.*, 1998). We therefore conclude that the mutant Pro207Ala exhibits the same octameric quaternary structure as the wild type enzyme and that the overall fold of the enzyme was not changed by the mutation.

Additional molecular modeling studies indicate the primary source of structural change to be a transition of the peptide bond torsional angle ω linking residues 206 and 207 from the *cis* to the *trans* configuration. There are a few further theoretical considerations favouring a change from the *cis* to the *trans* peptide bond upon mutation. A potential stabilization of the Trp206–*cis*Pro207 dipeptide in creatine kinase might come from hydrophobic stacking between the aromatic and proline rings, which is impossible in the *trans*-conformation and which has been suggested in a similar way for the NH_2-Phe-*cis*Pro-COOH dipeptide (Hetzel and Wüthrich, 1979). This interaction is impossible in the Pro207Ala mutant, therefore no stabilization of the *cis* peptide bond is expected, making the energetically more favoured *trans* peptide bond more likely to be formed. Quite recently, a similar example of a Trp–*cis*Pro bond has been shown in the CD1 molecule (B.Segelke, personal communication). In addition, a search for the occurrence of *cis* peptide bonds in the protein databank yielded a few examples of non-proline *cis* amino acids, none of them, however, being alanine. Furthermore non-proline amino acids in a *cis* configuration are almost always found to be stabilized by some electrostatic interaction with neighbouring residues, quite unlikely for alanine.

The occurrence of a *cis* proline in Mi_b-CK gives rise to a fundamental question as to why and how a *cis* peptide bond can exist in a mitochondrial protein, located in the intermembrane space, in the first place. An important implication comes from the fact that the protein studied is located in the mitochondrial intermembrane space: proteins are imported into this subcellular compartment in a fully unfolded form (Attardi and Schatz, 1986; Pfanner and Neupert, 1990). In the absence of ordered structure, as in unfolded proteins, the *trans*-isomer of an Xaa-Pro peptide bond (with Xaa denoting any amino acid) is favoured over the *cis* (Cheng and Bovey, 1977; Gratwohl and Wüthrich, 1981). It is unclear, however, whether and how a *trans* to *cis* isomerization of the peptide bond can occur within the mitochondrial intermembrane space. Mitochondrial prolyl *cis-trans* isomerase activity has been found in *Saccharomyces cerevisiae* (Rospert *et al.*, 1996) and cyclophilin from rat liver mitochondria has been shown to possess prolyl *cis-trans* isomerase activity (Nicolli *et al.*, 1996). These proteins, however, have been localized in the mitochondrial matrix rather than the intermembrane space where Mi_b-CK is located.

Another possibility is to assume that at least some peptide bonds can be in the *cis*-range in unfolded proteins. A statistical mechanics investigation of unfolded phosphoglycerate kinase (Calmettes *et al.*, 1993) and a recent unfolding study of creatine kinase (Forstner, 1996) have shown that locally organized peptide stretches are likely to exist in unfolded proteins. It is conceivable that such structured patches also bear prearranged *cis*-prolines, stabilized for example by neighbouring aromatic residues as mentioned above.

We have shown that the mutation of an uncharged residue far from the active site to another uncharged residue can have effects on the active site without disturbing the overall structure of the protein, the most probable change in the structural organization of the protein being a change from a *cis* to a *trans* peptide bond upon mutation. As *cis*-peptide bonds are thermodynamically disadvantaged over the respective *trans*-bonds, their existence has to provide some means of overall advantage for the proteins that bear them, either by directly organizing local structure that is important for biological function or, as suggested here for creatine kinase, by providing indirect stabilization of electrostatic contributions to the active site. As creatine kinase has been suggested to be a member of the 'conzyme' class of enzymes (Stroud, 1996), for which in the meanwhile some evidence has accumulated both experimentally (Forstner *et al.*, 1997) and by modeling (Forstner *et al.*, unpublished) subtle contributions to the electrostatics of the active site may indeed be of great importance for ensuring the high catalytic rate of this important enzyme in energy metabolism. A final explanation of the presumably subtle structural changes leading to the pronounced effects on enzymatic activity will have to await elucidation of the mutant's X-ray structure which is currently being pursued.

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