Dimer−Dimer Interactions in Octameric Mitochondrial Creatine Kinase†

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ABSTRACT: Mitochondrial creatine kinase (Mi-CK) forms octamers and dimers, which are readily interconvertible *in vitro*. The kinetic and thermodynamic octamer stability of wild-type and two mutant, octamer-destabilized forms of chicken sarcomeric Mi-CK was investigated at varying temperatures, pHs, and salt and substrate concentrations, in order to identify parameters which might regulate the octamer/dimer ratio *in vivo* and to assess the nature of octamer-stabilizing interactions. For wild-type Mi-CK, the rate of the transition state analogue complex (TSAC)-induced octamer decay increased with increasing temperature up to 28 °C; increasing pH markedly accelerated the decay in a biphasic manner. The substrate-dependent decay data suggest that also the productive enzymatic transition state of Mi-CK induces an octamer-destabilizing conformation. Thermodynamically, the octamers are stabilized by a combination of hydrophobic and polar contributions. Van't Hoff analysis showed that hydrophobic interactions dominate both in the absence of substrates and in the TSAC conformation, since the equilibrium octamer fractions increased with increasing temperatures, in spite of the accelerated decay kinetics. For the Mi-CK mutant E4Q, a similar temperature dependence was found; in contrast, mutant W264C exhibited an inverted temperature dependence, suggesting that hydrophobic interactions might be largely abolished in this mutant. Both the kinetic and the thermodynamic data seem to suggest that the octamer−dimer transitions of Mi-CK might not play a major role in a fast regulation of mitochondrial energy metabolism, but could rather be involved in slow long-term modulations.

Mitochondrial creatine kinase (Mi-CK),[1] an enzyme found in the mitochondrial intermembrane space of higher animals, fulfills several key functions in the energy metabolism of tissues with high and fluctuating energy requirements [for reviews, see Wyss et al. (1992) and Wallimann (1994)]. In contrast to the cytosolic CK isozymes, which are exclusively dimeric, Mi-CKs form both octameric and dimeric structures. It has been shown that octameric Mi-CK is preferentially suited for lipid binding and that it can even induce contact site formation between mitochondrial lipid membranes (Rojo et al., 1991a,b). Therefore, the oligomeric state of Mi-CK might play an important role in the adaptation of energy metabolism by regulating the formation of contact sites between the outer and inner mitochondrial membranes; such a dynamic formation of contact sites has been shown to occur in cells with varying energy demands [reviewed in Brdiczka (1991)]. The fact that the thermodynamic and kinetic octamer stabilities of Mi-CKs from different tissues and species display significant variability (Wyss et al., 1992) suggests that octamer stability may indeed correlate with the specific energy demands of a certain cell type.

Several parameters which could influence Mi-CK octamer stability *in vivo* have been investigated previously (Schlegel et al., 1988, 1990). The proportion of octamers was found to increase with Mi-CK concentration as a consequence of mass action, and it was shown qualitatively that high pH and the presence of organic solvents like ethylene glycol destabilize the octameric structure. Benzamidine and diaminobenzidine were found to exert an octamer-stabilizing influence of unknown origin (Marcillat et al., 1987), and adenine nucleotides showed a tendency to dissociate Mi-CK octamers (Marcillat et al., 1987). The most striking effect is produced by a mixture of MgADP, nitrate, and creatine (Cr), which induces the formation of a transition state analogue complex (TSAC) with CK (Milner-White & Watts, 1971); addition of such a TSAC substrate mixture to octameric Mi-CK results in octamer dissociation within several minutes (Marcillat et al., 1987; Gross & Wallimann, 1993), whereas in the absence of substrates, Mi-CK dissociates only with half-life times of hours to weeks, depending on the Mi-CK isoform and the conditions employed (Schlegel, 1989). In order to judge which factors might act as regulators of the octamer/dimer equilibrium *in vivo*, we now quantitatively determined the effects of temperature, pH, and substrate concentrations on the kinetics of octamer dissociation, employing a fluorescence-spectroscopic assay that has been described recently (Gross & Wallimann, 1993). Furthermore, we studied the influence of temperature and ionic strength on the octamer/dimer equilibrium of wild-type and two mutant, octamer-destabilized Mi-CKs to characterize the nature of dimer−dimer interactions within Mi-CK octamers.

MATERIALS AND METHODS

Chicken sarcomeric mitochondrial creatine kinase (Mi_b-CK) and the Mi_b-CK mutants E4Q (Kaldis et al., 1994; material kindly provided by P. Kaldis) and W264C (Gross et al., 1994) were overexpressed in *Escherichia coli* and

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[1] Abbreviations: Cr, creatine; EDTA, ethylenediaminetetraacetic acid; 2-ME, 2-mercaptoethanol; Mi-CK, mitochondrial creatine kinase; Mi_b-CK, chicken sarcomeric mitochondrial creatine kinase; PCr, phosphocreatine; TSAC, transition state analogue complex (Mi-CK·Cr·MgADP·nitrate).

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purified as described elsewhere (Furter et al., 1992). The proteins were stored at 4 °C as concentrated stock solutions (≥5 mg/mL); all enzymes were at least 90% octameric at these conditions. Protein concentrations were determined by the Bio-Rad assay (Bradford, 1976), using BSA as standard. Apyrase (grade VII) was purchased from Sigma.

Kinetic Studies. Mi-CK octamer dissociation kinetics were measured by the fluorescence-spectroscopic assay as described earlier (Gross & Wallimann, 1993), monitoring the decrease in intrinsic protein fluorescence at excitation and emission wavelengths of 295 and 340 nm, respectively. A SPEX Fluorolog-2 fluorescence spectrometer, equipped with a 450-W xenon arc lamp, was used with 1-cm cuvettes (rectangular excitation); excitation and emission slit widths were 0.2 and 5 mm, respectively, and the sample holder was thermostated by an external heating/cooling bath. The protein stock solution was diluted to a final assay concentration of 25 μg/mL, and octamer dissociation was induced by addition of a concentrated solution either of the complete standard TSAC mixture (final assay concentrations: 4 mM ADP, 5 mM $MgCl_2$, 20 mM Cr, 50 mM KNO_3) or, for the substrate-dependent measurements, of varying amounts of one component, together with all other components at the fixed standard concentrations. The standard buffer was 50 mM sodium phosphate, 150 mM NaCl, 2 mM 2-ME, and 0.2 mM EDTA (buffer A); the standard pH was 7.4, and the temperature 25 °C unless indicated otherwise. For the Mg-dependent experiments, EDTA was omitted from the buffer. For the pH-dependent study, the buffer was prepared with sodium dihydrogen phosphate, the approximate desired pH values were adjusted by addition of 1 M NaOH, and the actual pH was verified individually after each dissociation experiment. All kinetic experiments were repeated at least twice with similar results.

Octamer/Dimer Equilibrium Studies. To circumvent the dilemma that increasing temperatures tend to increase the octamer percentage (see Results), but low temperatures render octamer dissociation in the absence of substrates impracticably slow [e.g., for Mi_b-CK, dissociation at 4 °C and pH 7.2 displays a half-life time of 9.2 days (Kaldis et al., 1994)], we started our temperature-dependent equilibrium studies with TSAC-dimerized protein (standard TSAC mixture in buffer A, pH 7.0, 25 °C) and induced reoctamerization, which is comparatively fast (Gross & Wallimann, 1993), by the addition of apyrase (2 units/mL), thereby hydrolyzing the ADP to AMP + P_i. Complete octamer/dimer equilibration was achieved by overnight incubations of the TSAC-dissociated proteins with apyrase at defined temperatures, and equilibrium octamer/dimer distributions were determined by gel permeation chromatography on an FPLC Superose-12 column (Pharmacia) as described previously (Gross & Wallimann, 1993). The column was run at 4 °C with buffer A as the eluent (flow rate, 0.7 mL/min); since at 4 °C octamer dissociation in the absence of the TSAC substrates occurs in a time scale of days (see above), and since the samples were always diluted prior to chromatography, the octamer/dimer distribution was not affected by the analytical procedure. From the oligomeric distributions, the apparent overall equilibrium constants, K_{oct}, for octamer formation from dimers were calculated. In preliminary experiments, we determined that, at 22 °C, the negative $\Delta G°$ of octamer formation for Mi-CK in the presence of the apyrase-treated TSAC mixture was only about 5% decreased with respect to completely substrate-free conditions; therefore we conclude that such "apyrase" conditions (which will be termed quasi-substrate-free conditions in the following) can be used as an *in vitro* model for substrate-free Mi-CK. The temperature-dependent octamer stability of Mi-CK in the presence of the complete TSAC mixture was determined after overnight incubation of Mi-CK samples with the TSAC substrates (standard concentrations, see above; buffer A, pH 7.0) at defined temperatures. The salt-dependent equilibrium experiments were all performed without the use of TSAC/apyrase, since at 22 °C spontaneous octamer dissociation occurs sufficiently fast to achieve equilibrium by 1-week incubations of diluted octameric Mi-CK. All octamer/dimer equilibrium experiments were performed at least three times with similar results.

RESULTS AND DISCUSSION

Temperature Dependence of the Octamer Dissociation Rate. The temperature-dependent rate of the TSAC-induced Mi-CK octamer decay was measured over the range from 1 to 42 °C (pH 7.4) by monitoring the decrease in protein fluorescence as described previously (Gross & Wallimann, 1993). The corresponding first-order rate constants were obtained by fitting a single-exponential rate law to the fluorescence-spectroscopic decay curves. Although the absolute fluorescence intensity of the protein decreased upon raising the temperature, the relative fluorescence change upon octamer dissociation remained essentially constant over the temperature range assayed, and the decays were always single-exponential (Figure 1A). The dissociation rate continuously increased with increasing temperature between 1 and 28 °C (Figure 1B), the decay being about 3-fold faster at this optimum temperature than at 1 °C; above 28 °C, however, the dissociation rate sharply decreased again. The dependence of ln k on $1/T$ was linear between 1 and 28 °C (Figure 1B, inset), obeying the Arrhenius Law,

$$\ln k = \ln A - E_a/RT$$

where A represents the so-called preexponential factor (which cannot readily be interpreted in this case), and E_a is the activation energy of the reaction. For any elementary reaction, E_a must be positive; therefore, negative activation energies, as apparent in the case of the Mi-CK octamer dissociation above 28 °C, definitely indicate a composite process that involves more than a single elementary reaction. A negative Arrhenius activation energy can result from a fast, exothermic preequilibrium; this is probably the explanation for the observed decline of the dissociation rate of Mi-CK above 28 °C, since the binding of the TSAC substrates to the enzyme represents such a fast preequilibrium. In preliminary microcalorimetric titrations, it was found that the binding of the TSAC substrates to CK is in fact exothermic (not shown). It will be shown below that, in the standard TSAC mixture, two components are present at nonsaturating concentrations; therefore, it is likely that above 28 °C their binding to Mi-CK indeed becomes rate-limiting for the dissociation reaction. However, from the linear part of the Arrhenius plot below 28 °C, a positive Arrhenius activation energy of 28.3 kJ/mol and a preexponential factor of 669 s^{-1} can be determined for the dissociation reaction, allowing the calculation of the expected dissociation rate at any temperature in this range under the given conditions.

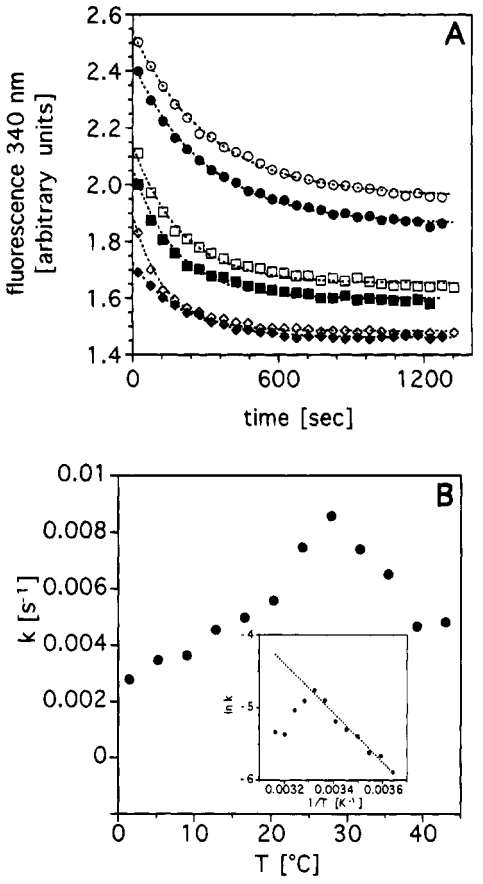

FIGURE 1: Temperature dependence of the TSAC-induced octamer decay. Octameric Mi-CK was dissociated at a protein concentration of 25 μg/mL in buffer A by addition of the standard TSAC mixture (4 mM ADP, 5 mM $MgCl_2$, 20 mM and Cr, and 50 mM KNO_3) at varying temperatures between 1 and 42 °C. First-order rate constants were obtained from the fluorescence traces by direct curve fitting. (A) Representative fluorescence traces of octamer decays (from top to bottom) at 4, 8, 16, 20, 32, and 42 °C, respectively (every tenth data point shown). The dashed lines represent single-exponential curve fits. (B) Plot of the temperature-dependent rate constants. Inset: Same data shown as an Arrhenius plot, with linear regression of data points below 30 °C (dashed line).

Influence of pH on Octamer Dissociation. The rate of the TSAC-induced octamer decay was determined at 25 °C over a pH range from 5.0 to 8.9. Due to the pH adjustments, the sodium concentration increased from 150 mM at pH 5.0 to about 200 mM at pH 8.9. Control decay experiments with various NaCl concentrations showed that this small difference in ionic strength did not significantly affect the dissociation rate (not shown). The proton concentration was found to strongly influence the kinetic stability of the Mi-CK octamer (Figure 2). Below pH 5.5, almost no dissociation took place; when the pH was raised above this value, the dissociation rate initially increased moderately; and between pH 7 and 8, a very steep increase of the rate constant, with a midpoint at approximately pH 7.5, was observed. The maximum dissociation rate (0.016 s^{-1}) was achieved at pH 8.0. At pH values beyond 8.0, the dissociation rate decreased again (not shown); however, the values above pH 8.0 were not reliable, since magnesium salts partly precipitated due to the alkaline conditions.

It has been shown before that also, in the absence of substrates, both the kinetic (Schlegel et al., 1990) and the thermodynamic octamer stability of Mi-CK decrease with increasing pH (Schlegel, 1989), indicating that the deprotonation of ionizable groups directly weakens the interdimer interactions. The midpoints of the two phases of the pH-dependent increase of the rate of the TSAC-induced octamer dissociation suggest that at least two ionizable groups with pK_a values of roughly 6.2 and 7.5, respectively, play a role in this reaction. Both pK_a values would suggest that the protonation/deprotonation of histidine and/or cysteine residues is rate-determining for the TSAC-induced octamer decay. Since in this process not only the dimer−dimer interactions themselves but also the binding of substrates and the TSAC-induced conformational change must be considered as potential pH-dependent rate-limiting reactions, one cannot clearly judge whether both observed pK_a values can be attributed to groups that directly participate in inter-dimer contact formation. From enzyme kinetic studies on M-CK, it is assumed that a histidine residue with a pK_a of about 7.0 is involved in acid/base catalysis (Cook et al., 1981); therefore, one could speculate that this group might be involved in the TSAC-induced conformational change as well. However, on the basis of the equilibrium data on the pH-dependent octamer stability in the absence of substrates (Schlegel, 1989), one can infer that at least one of the two observed pK_a values should correspond to groups directly involved in octamer formation.

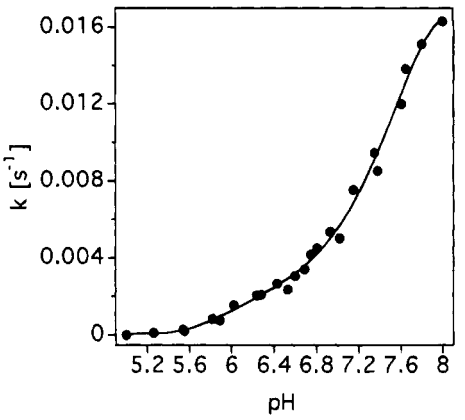

FIGURE 2: pH dependence of the TSAC-induced octamer decay. Octamer dissociation rates were determined fluorescence-spectroscopically at varying pH values (25 °C), using the standard TSAC mixture. (Above pH 8, no reliable data points were obtained due to magnesium salt precipitation).

Dependence of the Octamer Dissociation Rate on the Concentrations of Individual TSAC Components. Varying the concentration of one TSAC component at fixed concentrations of all other components (standard concentrations, see Materials and Methods) should open the possibility to assess the binding behavior of the component of interest to the enzyme by measuring the resulting octamer decay rates. This was done for all four individual components of the TSAC mixture (ADP, Cr, Mg^{2+}, and KNO_3). All experiments were performed at 25 °C, using the standard buffer A at pH 7.4. Since the decay rate should be proportional to the number of Mi-CK octamers in the TSAC conformation, the substrate-dependent rate constants should obey a Michaelis−Menten-type equation,

$$k_1 = k_{1,max}\,[S]^h/(K_m + [S]^h)$$

where h represents an optional Hill coefficient that can be introduced to account for possible cooperativity phenomena.

The dependence of the dissociation rate on the ADP concentration is shown in Figure 3A. The data could be

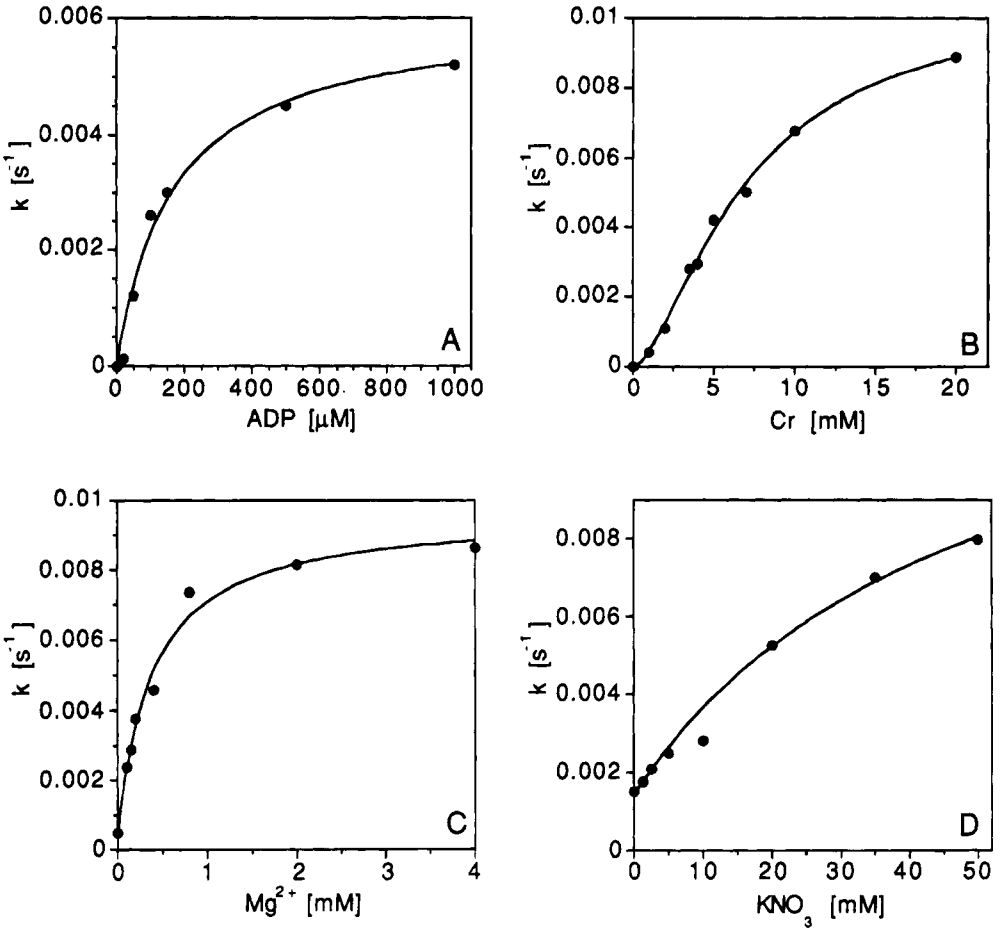

FIGURE 3: Dependence of octamer dissociation rates on the concentration of individual TSAC components. Octamer dissociation rates were measured fluorescence-spectroscopically at 25 °C and pH 7.4, using varying concentrations of ADP (A), Cr (B), $MgCl_2$ (C), or KNO_3 (D), keeping all other components fixed at the concentrations of the standard TSAC mixture (i.e., 4 mM ADP, 5 mM $MgCl_2$, 20 mM Cr, and 50 mM KNO_3). Lines represent curve fits to the Michaelis–Menten-type equations given in Results.

satisfactorily fit to the above equation, yielding a K_m for ADP of 166 ± 32 μM. This value is close to the upper limit of the enzyme kinetic K_m values for ADP reported in the literature [15–150 μM, summarized in Wyss et al. (1992), indicating that the binding mode of ADP in the TSAC is very similar to the formation of the productive enzyme–substrate complexes.

Figure 3B shows the effect of varying Cr concentrations on the octamer decay rate. Remarkably, the Cr-dependent curve of rate constants exhibited a sigmoidal shape, suggesting a cooperative process. For a satisfactory curve fit to the above Michaelis–Menten equation, a Hill constant of 1.6 ± 0.1 had to be introduced, yielding a K_m for Cr of 22.3 ± 3.3 mM. The K_m determined by this method, like the one for ADP, is of the same order of magnitude as the published enzyme kinetic value [8.9 mM (Furter et al., 1993)], suggesting that also the binding affinity of Cr to the TSAC is similar to that in the formation of the productive ternary complex. The observed cooperativity phenomenon can be interpreted in two ways: either the binding of Cr to the TSAC exhibits positive cooperativity (e.g., due to a communication of the two active sites of a dimer) or a critical "threshold" number of active sites per octamer has to be in the TSAC conformation in order to induce dissociation. The first interpretation is the less likely one, since a positively cooperative binding of individual substrates has never been observed for CK. Thus, we would rather favor the second explanation, which is also consistent with the finding that intermediate Mi-CK oligomers (e.g., tetramers) are extremely unstable (Gross & Wallimann, 1993; Kaldis et al., 1994), such that the breakage of only a few inter-dimer contacts within an octamer could indeed induce full dissociation.

The Michaelis–Menten curve for the Mg^{2+}-dependent octamer dissociation rate is shown in Figure 3C. Even in the absence of externally added Mg^{2+}, some octamer dissociation was observed. This is probably due to small contaminations of metal ions in the buffer and in the glassware, since after addition of 0.5 mM EDTA no octamer decay occurred any more (not shown). The unknown "baseline" metal concentration, X, was introduced into the Michaelis–Menten equation as an additional fitting parameter, by which the nominal Mg^{2+} concentration was corrected:

$$k_1 = k_{1,max}([S] + X)/(K_m + [S] + X)$$

The experimental data could be satisfactorily fitted to this modified equation resulting in a K_m for Mg^{2+} of 363 ± 66 μM and a "baseline" metal concentration equivalent to 17 μM Mg^{2+}. In CK, the divalent metal ion is known to be only *indirectly* bound to the enzyme via its complex with the phosphate groups of the nucleotide (Cohn & Leigh, 1962; O'Sullivan & Cohn, 1966). Thus, the K_m for the metal ion should be closely coupled to the K_m for ADP; since the MgADP stability constant is on the order of 3000–4000 M^{-1}

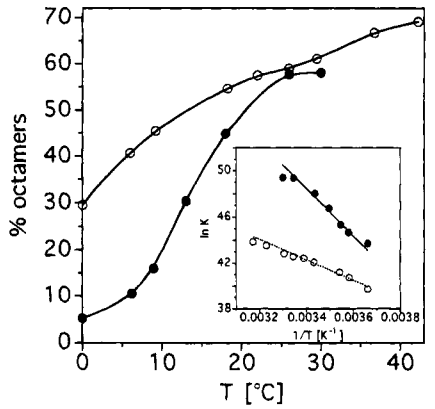

FIGURE 4: Temperature dependence of the Mi-CK octamer/dimer equilibrium. To achieve octamer/dimer equilibrium at quasi-substrate-free conditions (●), octameric Mi-CK at a concentration of 0.1 mg/mL was dissociated by the TSAC mixture before apyrase was added to hydrolyze the ADP at defined temperatures. Oligomeric equilibrium in the presence of the complete TSAC substrates (○) was obtained by overnight incubation of octameric Mi-CK at a protein concentration of 1 mg/ml with the TSAC mixture at the temperatures indicated. Octamer percentages were determined by gel permeation chromatography. Inset: van't Hoff plot of the data with linear regressions.

(O'Sullivan & Smithers, 1979), the added Mg^{2+} will not be stoichiometrically bound to the nucleotide. Also, at pH 7.4, hydroxide and phosphate ions probably act as competing ligands for Mg^{2+} binding, such that the observed K_m for Mg^{2+}, being about 2-fold higher than for ADP, appears reasonable.

To determine the K_m for nitrate ions, a baseline correction similar to that with the Mg^{2+}-dependent decay rates had to be performed in order to account for the slow octamer dissociation due to chloride ions that was observed even in the absence of nitrate. A plot of the nitrate-dependent dissociation rates, together with the least-squares fit to the modified Michaelis–Menten equation, is shown in Figure 3D. From the curve fit, a K_m for nitrate of 43.5 ± 6.3 mM was obtained; the basal chloride concentration of 150 mM was calculated to equal the effect of an additional 4.8 mM nitrate.

The above results indicate that in the standard TSAC mixture two components are present at subsaturating concentrations, namely, Cr (K_m = 22 mM, 20 mM in the standard mixture) and nitrate (K_m = 44 mM, 50 mM in the standard mixture); the remaining two components can be assumed to be sufficiently concentrated for enzyme saturation. This implies that the true K_m values for ADP and Cr that would be measured at saturating concentrations of all other substrates are probably slightly lower than determined here, since the binding of the creatine and nucleotide substrates to CK is synergistic (Furter et al., 1993).

Temperature Dependence of the Octamer/Dimer Equilibrium. To study the temperature dependence of the octamer/dimer equilibrium, TSAC-dimerized Mi-CK at a protein concentration of 0.1 mg/mL was reoctamerized at different temperatures by the addition of apyrase, to give quasi-substrate-free conditions (see Materials and Methods). Increasing temperatures between 0 and 30 °C were found to stabilize the octameric structure (Figure 4, closed circles). On first sight, such an effect seems surprising since, due to the gain in entropy that is brought about by dissociation reactions in general, an octamer *decay* should be expected to be favored at increasing temperatures. The observed inverse temperature dependence can only be explained by a large contribution of hydrophobic interactions, which are predominantly entropy-driven, to the octamer-stabilizing forces. In fact, hydrophobic interactions are known to play a central role in intersubunit contacts of proteins (Jaenicke & Rudolph, 1986). Above 30 °C, the stability of the Mi-CK octamers did not increase further with increasing temperature. At the highest temperatures assayed, i.e., 37 and 42 °C (not shown), an apparent decrease in octamer percentage was observed; however, this was probably due to aggregation of the protein, since the total amount of protein eluting from the gel filtration column was significantly reduced, indicating that a significant fraction of the protein had not passed the injection prefilter (0.2-μm exclusion size). Also, an additional monomer peak was seen at 42 °C; therefore, the data points at 37 and 42 °C were omitted from quantitative evaluation.

Table 1: Thermodynamic Parameters of the Mi-CK Octamer/Dimer Equilibrium[a]

	$\Delta G°_{oct}$ (4 °C) [kJ/mol]	$\Delta G°_{oct}$ (20 °C) [kJ/mol]	$\Delta H°_{oct}$ [kJ/mol]	$-T\Delta S°_{oct}$ (20 °C) [kJ/mol]
wild type Mi-CK	−86.3	−100.9	166.5	−267.9
wild type Mi-CK + TSAC	−77.9	−87.1	81.3	−168.4
Mi-CK E4Q	−71.9	−85.0	155.8	−240.8
Mi-CK W264C	−87.8	−84.5	−144.9	60.4

[a] The free energies and enthalpies of octamer formation were derived from van't Hoff plots of the temperature-dependent octamer/dimer equilibrium of wild-type Mi-CK and mutants E4Q and W264C (Figures 4 and 5). $\Delta H°$ values are only valid in the linear range of the van't Hoff plots (0–30 °C, wild type; 0–20 °C, mutants); the entropy term ($-T\Delta S°$) was calculated by subtraction of $\Delta H°$ from $\Delta G°$.

When the octamer stability data were plotted according to the van't Hoff relation, a negative linear correlation between ln K_{oct} and $1/T$ between 0 and about 30 °C was found (Figure 4, inset), indicating that the association enthalpy is constant and strongly positive in this temperature range. Calculation of the thermodynamic parameters (Table 1) from the linear part of the plot revealed that the positive $\Delta H°$ of octamer formation must be counterbalanced by a very large negative $T\Delta S°$ term to account for the favorable free energy of the association reaction. Such a large entropy contribution can indeed only be provided by hydrophobic interactions at the dimer–dimer interfaces.

In the presence of the TSAC mixture (Figure 4, open circles), about 10-fold higher protein concentrations were required to obtain octamer percentages similar to these observed under quasi-substrate-free conditions. The temperature dependence of the octamer/dimer equilibrium was similar to that of the apyrase-treated samples, but in contrast to the latter, temperatures above 30 °C neither caused a drop in octamer percentage nor led to partial aggregation or monomerization. A similar substrate protection has been previously observed for the Mi-CK mutant W206C (Gross et al., 1994), which was also significantly stabilized in the presence of the TSAC mixture.

The energetic data (Table 1) derived from the van't Hoff plot (Figure 4, inset) indicate that also, in the presence of the TSAC substrates, Mi-CK octamer formation is strongly

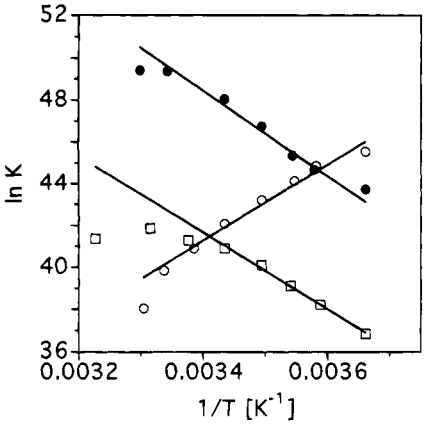

FIGURE 5: Temperature-dependent octamer/dimer equilibrium of Mi-CK mutants E4Q and W264C. The temperature-dependent octamer/dimer equilibria of Mi-CK mutants E4Q (□) and W264C (○) at quasi-substrate-free conditions were determined as described for Figure 4 and compared to the corresponding wild-type data (●). The data are given as van't Hoff plots, the lines representing linear regressions of the linear parts of the plots.

entropy-driven, since a positive $\Delta H°$ is compensated by a larger negative $T\Delta S°$.

Energetics of the Octamer/Dimer Equilibrium for Mi-CK Mutants E4Q and W264C. In two previous studies (Gross et al., 1994; Kaldis et al., 1994), mutant Mi-CKs with significantly destabilized octamers were identified. One type of mutants contained single or multiple replacements of charged N-terminal residues (Kaldis et al., 1994), while another mutant had Trp-264 replaced by cysteine (Gross et al., 1994). Neither of the mutants exhibited major alterations in enzyme kinetic parameters, indicating that no significant changes in the overall tertiary structure had occurred. In the present study, the octamer stability of Mi-CK mutants E4Q (as an example for the N-terminal mutants) and W264C was investigated in more detail. The temperature dependence of the octamer/dimer equilibrium for the two mutants at quasi-substrate-free conditions is shown as a van't Hoff plot in Figure 5. As for the wild-type enzyme in the presence of the TSAC mixture, protein concentrations of around 1 mg/mL were required to obtain measurable amounts of octamers, reflecting a marked octamer destabilization for both mutants. However, the temperature dependence of the octamer stability of the two mutants showed a striking divergence. While in the range 0−20 °C mutant E4Q still exhibited a temperature dependence similar to that of the wild-type protein, the octamer stability−temperature relationship was completely inverted for mutant W264C. The energetic parameters (Table 1) illustrate the details of the alterations caused by the mutations. For mutant E4Q, where a negatively charged residue (Glu) of the N-terminus has been replaced by an uncharged residue of the same size (Gln), the association enthalpy was slightly less unfavorable, and the stabilizing entropy contributions were reduced by about 10%. Although this result does not allow a straightforward interpretation, it is consistent with a weakening of ionic interactions, as proposed by Kaldis et al. (1994), since ion pair formation can be weakly entropy-driven (Cantor & Schimmel, 1980). This view is further supported by the salt-dependent data shown below.

For mutant W264C, where a putative dimer−dimer interface Trp residue (Gross & Wallimann, 1993; Gross et al., 1994) has been replaced by a small, polar residue (Cys), the energetic parameters (Table 1) reveal an inversion of the relation between entropic and enthalpic contributions to the stability of the Mi-CK octamer. In the wild-type protein, a positive $\Delta H°$ value opposes octamer formation, but is overcome by the highly negative value of $T\Delta S°$ that is provided by the "hydrophobic effect". This principle of enthalpy−entropy compensation is frequently encountered when dealing with intra- and inter-protein interactions, and it is valid for most subunit associations (Cantor & Schimmel, 1980). The drastic effect of mutation W264C, being reflected by the inversion of the signs for $\Delta H°$ and $-T\Delta S°$, suggests that the hydrophobic part of the dimer−dimer interactions might be largely abolished for this mutant. However, one Trp residue alone is unlikely to form a functional hydrophobic interaction surface; therefore, the severe effect of the single Trp replacement must be due either to a sterical distortion of a major interaction surface, interfering with the optimized fit of the two complementary contact regions, or to the wild-type interface containing just the minimum amount of hydrophobic residues necessary for a cooperative interaction between two dimers. If one assumes as a simplification that the entire hydrophobic inter-dimer interaction is abolished with mutant W264C, and that the complete difference in $T\Delta S°$ (328 kJ/mol) compared to the wild type is due to this loss of hydrophobic interaction, one can try to estimate the size of the total hydrophobic interaction area. Using the approximate $T\Delta S°$ value of −25 cal/mol per 1 Å^2 of hydrophobic interaction surface proposed in Cantor and Schimmel (1980), around 3100 Å^2 of hydrophobic surface should become buried upon octamer formation. Adopting a simplified model with the Mi-CK octamer representing an ideal cube of 100 Å side length (Gross & Wallimann, 1993), the maximum area that could theoretically become buried is 40 000 Å^2; this simple consideration shows that only a small fraction (less than 10%) of the theoretically available dimer surface would actually participate in hydrophobic dimer−dimer contacts. This in turn supports the hypothesis that the size of the hydrophobic contacts might indeed be just sufficient for a stable interaction, such that the removal of one critical residue could cause dissociation. Recent preliminary low-resolution X-ray data (T. Schnyder and W. Kabsch, unpublished results) and electron-microscopic data from two-dimensional crystallization (Schnyder et al., 1994) have shown that octameric Mi-CK indeed displays a rather "fragile" structure, with only few dimer−dimer interaction sites.

Influence of Salt on the Octamer/Dimer Equilibrium. The NaCl-dependent octamer percentages of Mi-CK at 22 °C in the absence of substrates are shown in Figure 6A. For the wild-type protein, increasing NaCl concentrations up to 1 M resulted in a successive shift of the oligomeric equilibrium toward the dimeric form. However, above 1 M NaCl, this dissociating effect ceased; even at 2 M NaCl, complete dimerization could not be achieved. In Figure 6B, the data are plotted as ln K_{oct} versus the square root of the ionic strength. In the case of a purely ionic interaction, according to the Debye−Hückel theory, such a plot should be linear, the slope being proportional to the number of interacting charges. The marked deviation of the wild-type data from linearity, however, suggests that the dimer−dimer interactions are a combination of both polar and hydrophobic contributions: at low NaCl concentrations, the dissociating salt effect predominates, but at higher ionic strength, the

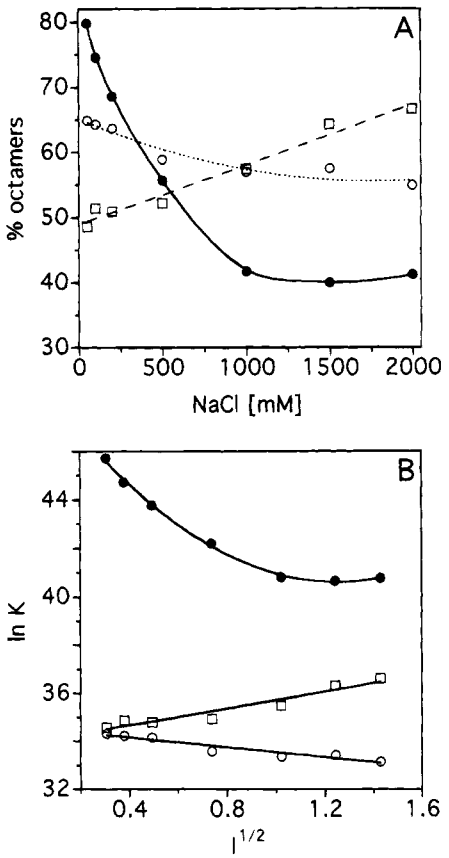

FIGURE 6: Salt dependence of the octamer/dimer equilibrium in the absence of substrates. Wild-type Mi-CK (closed circles) and Mi-CK mutants E4Q (open squares) and W264C (open circles) were incubated for 6 days at 22 °C in 25 mM sodium phosphate buffer, pH 7.0, supplemented with increasing NaCl concentrations. Protein concentrations were 0.1, 1, and 2 mg/mL for the wild-type protein and mutants E4Q and W264C, respectively. Octamer percentages were determined by gel permeation chromatography. (A) Direct plot. (B) Plot of ln K vs the square root of the ionic strength, according to the Debye–Hückel theory.

stabilizing influence of salt on the hydrophobic interactions increasingly compensates this destabilization. However, to account for the relatively steep salt-dependent destabilization at low NaCl concentrations, an additional mechanism of NaCl action on the octamer/dimer equilibrium might be considered. It is known that chloride binds to the active site of CK, and like nitrate, it can support the formation of transition state analogue complexes (Milner-White & Watts, 1971). Therefore, chloride might specifically destabilize Mi-CK octamers by binding to the active site.

In contrast to the wild-type enzyme, the two Mi-CK mutants E4Q and W264C, in addition to their general octamer destabilization, did not show such a biphasic response to increasing NaCl concentrations (Figure 6a/b). Mutant E4Q appeared to be weakly stabilized by increasing amounts of NaCl, as reflected by the approximately linear plot of ln K_{oct} versus $I^{1/2}$, suggesting that the hydrophobic part of the dimer–dimer interactions has become more dominant in this mutant. This strongly supports the view that Glu-4, and probably also some other charged residues in the N-terminal region of Mi-CK, indeed could be directly involved in octamer stabilization, as suggested by Kaldis et al. (1994). For mutant W264C, a weakly dissociating salt effect, giving an approximately linearly declining plot of ln K_{oct} versus $I^{1/2}$ over the whole range of [NaCl] assayed, was observed. Together with the above temperature-dependent data, this indicates that polar interactions indeed have gained importance for the octamer stability of this mutant, probably as the result of a drastic reduction of hydrophobic interactions.

Role of Hydrophobic and Polar Interactions in Octamer Stabilization: Relevance to the Function of Mi-CK in Vivo. Our data demonstrate that the octameric structure of Mi-CK is stabilized by a combination of hydrophobic and polar interactions, the contributions of which are illustrated in particular by the two Mi-CK mutants W264C and E4Q, respectively. Both for the wild-type enzyme and for mutant W264C, salt exerts only a relatively weak influence on octamer stability. This suggests that polar interactions do not play a fundamental role in octamer formation, but that they might have a rather modulating function, whereas hydrophobic contacts probably provide the basic stability of the octameric structure, in analogy to most other oligomeric proteins (Cantor & Schimmel, 1980). The different tissue- and species-specific Mi-CK sub-isoforms, which display remarkably variable octamer stabilites (Wyss et al., 1992), have significantly diverse N-terminal sequences, whereas the residues around Trp-264 are nearly invariant and specific for all Mi-CKs (Mühlebach et al., 1994). This suggests that isozyme-specific modulations of octamer stability could indeed be brought about by the different N-terminal sequences. A weakening of the polar interactions formed by N-terminal residues might also be primarily responsible for the TSAC-induced octamer destabilization, since the temperature-dependent octamer stability profile of mutant E4Q (Figure 5) is quite similar to the one observed for the wild-type protein in the presence of the TSAC substrates (Figure 4).

The kinetic and thermodynamic data on the octamer/dimer equilibrium of wild-type Mi-CK suggest that *in vivo* octamer–dimer interconversions probaby do not take place to a large extent and therefore are rather unlikely to play a major role in metabolic regulation *per se*. Firstly, no physiological effector has been found yet that could induce a rapid octamer dissociation comparable to the nonphysiological TSAC-induced reaction; the fastest observed *in vitro* octamer dissociation in the presence of the TSAC mixture (i.e., at pH 8.0) exhibited a half-life time of 43 s (Figure 2), which is still too slow to allow for a fast modulation of mitochondrial energy metabolism. Second, the concentrations of Mi-CK in the intermembrane space are supposed to be rather high [estimates range between 3.5 and 17 mg/mL; reviewed in Wyss et al. (1992)], such that the vast majority of the enzyme should be octameric at any conditions, and the high body temperature of 42 °C (chicken) also would rather favor the octameric state. However, small, long-term changes in the octamer/dimer distribution could be induced *in vivo* by changes in intermembrane space pH, volume (protein concentration), and membrane potential. The latter parameter, the influence of which is difficult to determine *in vitro*, could also have an accelerating effect on the kinetics of dissociation; therefore, a regulatory function of the octamer/dimer equilibrium on the coupled process of mitochondrial ATP production and PCr export still could be possible.

Our preferred assumption is that under physiological conditions the octameric Mi-CK switches into a relaxed conformation upon forming the productive transition state, thereby *transiently* weakening part of the dimer–dimer

interactions. However, this short-term destabilization would probably not be sufficient to dissociate the protein to a large extent; only in the case when the enzyme is artifically trapped in the transition state (i.e., by the TSAC mixture) is there enough time for the remaining contacts to break, leading to the dissociation of a significant population of octamers. In the working enzyme, it is likely that the breakage of some dimer−dimer interactions is necessary to allow for a hinge-bending motion of domains that supposedly occurs during catalysis (Morris & Cartwright, 1990), since the octameric structure probably imposes sterical constraints that could limit or even prevent domain motions. From preliminary X-ray data (T. Schnyder & W. Kabsch, unpublished results), it appears that the two globular domains of a monomer lie within the plane of the "top" and "bottom" faces of the octamer, such that a hinge-bending motion probably alters the shape of the internal channel that runs through the octamer. The question of whether such changes in channel shape and/or diameter could play a supportive role in substrate exchange or even in an active pumping of metabolites will be an interesting subject of further research on Mi-CK octamer dynamics.

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