Determination of the Affinity of Each Component of a Composite Quaternary Transition-State Analogue Complex of Creatine Kinase†

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ABSTRACT: Recombinant rabbit muscle creatine kinase (CK) was titrated with MgADP in 50 mM Bicine and 5 mM $Mg(OAc)_2$, pH 8.3, at 30.0 °C by following a decrease in the protein's intrinsic fluorescence. In the presence of 50 mM NaOAc, but in the absence of added creatine or nitrate, MgADP has an apparent K_d of 135 ± 7 μM, and the total change in fluorescence on saturation ($\Delta\%F$) is 15.3 ± 0.3%. Acetate was used as the anion in this experiment because it does not promote the formation of a CK·MgADP·anion·creatine transition-state analogue complex (TSAC) [Millner-White and Watts (1971) *Biochem. J. 122,* 727−740]. In the presence of 80 mM creatine, but no nitrate, the apparent K_d for MgADP remains essentially unchanged at 132 ± 10 μM, while $\Delta\%F$ decreases slightly to 13.2 ± 0.3%. In the presence of 10 mM nitrate, but no creatine, the apparent K_d is once again essentially unchanged at 143 ± 23 μM, but the $\Delta\%F$ is markedly reduced to 4.2 ± 0.2%. The presence of both 10 mM nitrate and 80 mM creatine during titration reduces the apparent K_d for MgADP 10-fold to 13.7 ± 0.7 μM, and $\Delta\%F$ increases to 20.6 ± 0.3%, strongly suggesting that the simultaneous presence of saturating levels of creatine and nitrate increases the affinity of CK for MgADP and promotes the formation of the enzyme·MgADP·nitrate·creatine TSAC. When the fluorescence of CK was titrated with MgADP in the presence of 80 mM creatine and fixed saturating concentrations of various anions, apparent K_d values for MgADP of 132 ± 10 μM, 25.2 ± 1.3 μM, 18.8 ± 0.9 μM, 13.7 ± 0.7 μM, and 6.4 ± 0.7 μM were observed as the anion was changed from acetate to formate to chloride to nitrate to nitrite, respectively. This is the same trend reported by Millner-White and Watts for the effectiveness of various monovalent anions in forming the CK·MgADP·anion·creatine TSAC. On titration of CK with MgADP in the presence of 80 mM creatine and various fixed concentrations of $NaNO_3$, the apparent K_d for MgADP decreases with increasing fixed concentrations of nitrate. A plot of the apparent K_d for MgADP vs $[NO_3^-]$ suggests a K_d for nitrate from the TSAC of 0.39 ± 0.07 mM. Similarly, titration with MgADP in the presence of 10 mM $NaNO_3$ and various fixed concentrations of creatine gives a value of 0.9 ± 0.4 mM for the dissociation of creatine from the TSAC. The data were used to calculate K_{TDAC}, the dissociation constant of the quaternary TSAC into its individual components, of 3×10^{-10} M^3. To our knowledge this is the first reported dissociation constant for a ternary or quaternary TSAC.

Creatine kinase (CK,[1,2] EC 2.7.3.2) is a key enzyme in excitable tissues that require large energy fluxes, i.e., skeletal muscle, heart muscle, and brain (*1*−*3*). It catalyzes the readily reversible phosphorylation of creatine by MgATP to form phosphocreatine and MgADP. CK, found primarily in vertebrates, is a member of a larger family of phosphagen kinases that catalyze comparable reactions with other guanidino substrates. All phosphagen kinases show a high sequence homology and have apparently evolved from a common ancestral gene (*4*, *5*).

Since the first report of its purification from rabbit muscle more than 4 decades ago (*6*), CK has been the subject of numerous studies of its physicochemical properties and catalytic mechanism. In the first three decades, structure−function information about CK was obtained by enzyme kinetic, chemical modification, and spectroscopic studies (see refs *1* and *2* for reviews). The past 15 years have seen new approaches added to the arsenal for the attack on the structure−function relationships in CK and other guanidino kinases, including site-directed mutagenesis (*7*−*15*) and X-ray crystallography (*16*−*19*).

Millner-White and Watts (*20*) showed convincingly that previously observed anomalies in the enzyme kinetic analyses of CK were due to the formation of a quaternary CK·MgADP·anion·creatine complex in the presence of certain monovalent anions. They proposed that a transition-state

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[1] Abbreviations: CK, creatine kinase; TSAC, transition-state analogue complex; D, MgADP; C, creatine; A, monovalent anion, usually nitrate; $\Delta\%F$, change in the percent fluorescence of CK on saturation with MgADP.

[2] All CK residues are numbered using the sequence of rabbit muscle CK reported elsewhere (*10*, *15*).

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Scheme 1

Transition state	MgADP-----P-----Creatine	(1 species)
Bound substrates	MgATP . . . . . Creatine	(2 species)
TSAC	MgADP . . A . . Creatine	(3 species)

analogue complex (TSAC) was formed in which the anion occupied the position of the transferable phosphate during the transition state of the enzyme-catalyzed reaction. Subsequent spectroscopic studies (*21*, *22*) and a recent X-ray crystal structure of arginine kinase with a bound TSAC (*18*) have confirmed this hypothesis. The ability of creatine kinase to bind this composite transition-state analogue appears remarkable considering that the enzyme must assemble three separate species into one complex. From an entropic standpoint, this is an even taller order than the reaction itself, which involves the binding of only two substrates (Scheme 1).

Quenching of intrinsic CK tryptophan fluorescence by added ADP has been used to determine the affinity of the nucleotide for the enzyme (*23*). The residue that is quenched by nucleotide has been shown (*8*) to be equivalent to the fully conserved Trp227 in rabbit muscle CK. We now report the use of fluorescence quenching to determine directly the K_d for MgADP, and indirectly the K_d values for creatine and nitrate, from the CK·MgADP (D)·nitrate (A)·creatine (C) TSAC complex of creatine kinase. We have also used the data, along with previously reported kinetic constants, to determine K_{TDAC}, the dissociation constant for the TSAC into its individual components.

MATERIALS AND METHODS

Materials. Creatine hydrate, ADP, magnesium acetate tetrahydrate, and Bicine were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium acetate trihydrate and sodium nitrate were obtained from Mallinckrodt Chemical Co. (St. Louis, MO), sodium chloride was from Aldrich Chemical Co. (Milwaukee, WI), sodium formate was from Fisher Scientific (Fair Lawn, NJ), and sodium nitrite was a product of J. T. Baker Chemical Co. (Phillipsburg, NJ). Recombinant rabbit muscle creatine kinase was overexpressed in *Escherichia coli* and purified to homogeneity by methods described previously (*10*, *15*). The enzyme was judged to be >98% pure by SDS−PAGE on 10−20% gradient polyacrylamide gels from Owl Separation Systems (Portsmouth, NH).

Stock Solutions. Solutions of magnesium acetate (100 mM) and sodium acetate, sodium nitrate, sodium nitrite, sodium chloride, and sodium formate (all 1.00 M) were prepared in deionized distilled water, filtered through a 0.2 μm membrane filter, and stored at room temperature. Bicine buffer (62.5 mM, pH 8.3) and 100 mM creatine in this buffer were also filtered and stored at room temperature. Stock solutions of 50 mM MgADP, adjusted to pH ~8.5, were filtered, divided into aliquots appropriate for one experiment, and stored at −20 °C. Such solutions were stable for up to 3 months.

Fluorescence Measurements. All fluorescence measurements were made at 30.0 ± 0.1 °C using a SLM Aminco 8100 spectrofluorometer running version 2.00 Beta6 software. Excitation of tryptophan residues in CK was carried out at 292 nm and a slit width of 2 nm to minimize the inner filter effect of added ADP, and emission was measured at 330 nm using a 4 nm slit width. Each solution to be titrated with MgADP (5.00 or 50.0 mM stock) contained the following in an initial volume of 3.00 mL: 50 mM Bicine, pH 8.3; 5.00 mM $Mg(OAc)_2$; 0−80.0 mM creatine; 0−50.0 mM NaX (X = NO_3^-, NO_2^-, HCO_2^-, Cl^-); and sufficient NaOAc such that [NaX] + [NaOAc] = 50.0 mM.

Data Collection. After each subsequent aliquot of stock MgADP was added, the solution was allowed to equilibrate for approximately 1 min; then twenty 1 s readings were collected, stored, and subsequently averaged to give a final value of relative fluorescence intensity. MgADP was incrementally added to a final concentration of up to 4.5 mM, the readings were corrected for dilution, and the data from 1.5 to 4.5 mM MgADP were extrapolated to zero concentration to allow correction for the inner filter effect of added nucleotide.

Data Analysis. Sigma Plot 2000 was used to fit each set of data for a fluorescence quenching experiment to the following equilibrium binding equation (*24*):

$$F_f = F_0 - F_{t\Delta}[1/(2nE_T)]\{(nE_T + K_d + [\mathrm{MgADP}]) - ((nE_T + K_d + [\mathrm{MgADP}])^2 - 4nE_T[\mathrm{MgADP}])^{0.5}\}$$

where F_f is the relative fluorescence intensity from a given measurement, F_0 is the initial fluorescence intensity, $F_{t\Delta}$ is the total change in fluorescence on saturation of CK with MgADP, n is the number of independent binding sites (n = 1 per CK monomer), K_d is the dissociation constant for MgADP, and E_T is the concentration of enzyme. The data fitting yields calculated values for F_0, $F_{t\Delta}$, and K_d, ± standard deviation, and each F_f reading was divided by F_0 and multiplied by 100 to give the fluorescence as a percent of the calculated initial fluorescence.

Dissociation constants for creatine and nitrate from the TSAC were determined by fitting the data to the equation:

$$y = y_0 + ab/(b + x)$$

where y is the observed K_d for MgADP, y_0 is the K_d for MgADP from the TSAC, b is the K_d for the variable component, either nitrate or creatine, from the TSAC, a is the change in the apparent K_d for MgADP as the concentration of the variable component goes from zero to infinity, and x is the concentration of the variable component.

RESULTS

The quenching of protein intrinsic fluorescence occurs by one of two mechanisms, either collisional or static (*25*). In the former, a mobile quencher absorbs energy from an excited fluorophore by repeatedly coming into contact with the fluorophore via diffusional motion; in the latter, the quencher forms a reversible complex and is fixed near the fluorophore with concomitant quenching. Since nitrate has been reported to be a collisional quencher of indole derivatives (*26*), we examined the ability of all small molecules and ions used in this work to cause collisional quenching of creatine kinase fluorescence. Table 1 shows that both nitrate and nitrite give significant collisional quenching of the intrinsic fluorescence of the enzyme, while acetate, formate, and chloride give minimal quenching at the concentrations used. We thus chose to use 50 mM acetate, formate, and

Table 1: Effect of Anions on the Intrinsic Fluorescence of Rabbit Muscle Creatine Kinase[a]

	% fluorescence quenching at		
anion	10 mM	50 mM	100 mM
none[b]	0	0	0
OAc^-	0	1	2
HCO_2^-	0	1	3
Cl^-	1	4	7
NO_3^-	15	50	72
NO_2^-	−2[c]	60	92

[a] The initial solution contained 1 μM CK subunit in 5.0 mM $Mg(OAc)_2$ and 50 mM Bicine, pH 8.3, at 30.0 ± 0.1 °C. The anion was added as a 1.00 M solution of the sodium salt in deionized distilled water. [b] An equivalent volume of water was added, and the relative fluorescence intensity was corrected as described in the text. [c] The relative fluorescence increased to 102% of the control at 10 mM $NaNO_2$ (105% at 5 mM $NaNO_2$) and then decreased as shown.

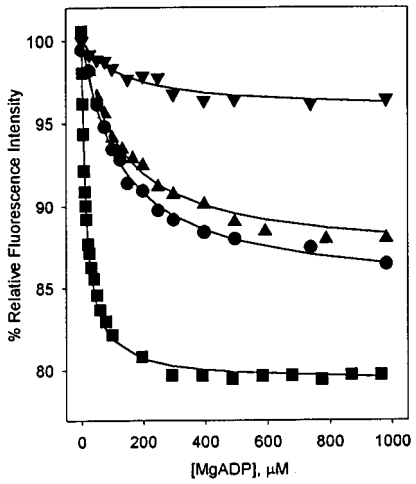

FIGURE 1: Effects of creatine and creatine plus nitrate on the fluorescence quenching titration of creatine kinase with MgADP. CK (1 μM) in 50 mM Bicine and 5 mM $Mg(OAc)_2$, pH 8.3, was titrated with MgADP at 30.0 °C in the presence of the following: (●) 50 mM NaOAc, $K_d = 135 \pm 7$ μM, $\Delta\%F = 15.3 \pm 0.3$; (▲) 80 mM creatine, 50 mM NaOAc, $K_d = 132 \pm 10$ μM, $\Delta\%F = 13.2 \pm 0.3$; (▼) 10 mM $NaNO_3$, 40 mM NaOAc, $K_d = 143 \pm 23$ μM, $\Delta\%F = 4.2 \pm 0.2$; and (■) 80 mM creatine, 10 mM $NaNO_3$, 40 mM NaOAc, $K_d = 13.7 \pm 0.7$ μM, $\Delta\%F = 20.6 \pm 0.3$. The data were corrected and the apparent K_d and $\Delta\%F$ calculated as described in the text.

chloride, but only 10 mM nitrate or nitrite, in the experiments described in the following paragraph.

The change in the intrinsic fluorescence of 1.0 μM creatine kinase subunit was followed as the enzyme was titrated with MgADP in 50 mM Bicine and 5 mM $Mg(OAc)_2$, pH 8.3, at 30.0 °C. The results (Figure 1) show that in the presence of 50 mM NaOAc, but in the absence of added creatine or nitrate, MgADP has a K_d of 135 ± 7 μM, and the total change in fluorescence on saturation is 15.3 ± 0.3%. Acetate was used as the anion in this experiment because it does not promote the formation of a TSAC (*20*). In the presence of saturating levels of creatine (80 mM), but no nitrate, the apparent K_d of MgADP remains essentially unchanged at 132 ± 10 μM, while $\Delta\%F$ decreases slightly to 13.2 ± 0.3%. These data confirm that when acetate is used as the anion, the K_d for MgADP is the same in the presence or absence of 80 mM creatine; i.e., acetate does not promote the formation of the TSAC. Likewise, in the presence of saturating nitrate (10 mM), but no creatine, the apparent K_d is still the same within experimental error at 143 ± 23 μM, although the $\Delta\%F$ is markedly reduced to 4.2 ± 0.2%. However, if saturating levels of both nitrate and creatine are present during titration, the apparent K_d of MgADP decreases an order of magnitude to 13.7 ± 0.7 μM, and $\Delta\%F$ increases to 20.6 ± 0.3%. The data show that the simultaneous presence of excess creatine and nitrate increases the affinity for MgADP and strongly suggests the formation of the enzyme·MgADP·nitrate·creatine TSAC.

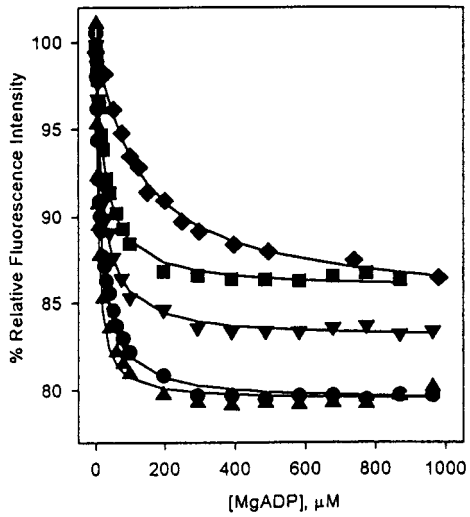

FIGURE 2: Effectiveness of various anions and saturating levels of creatine on the formation of a TSAC with creatine kinase as revealed by fluorescence quenching titration of creatine kinase with MgADP. CK (1 μM) in 50 mM Bicine, 5 mM $Mg(OAc)_2$, and 80 mM creatine, pH 8.3, was titrated with MgADP at 30.0 °C in the presence of the following: (◆) 50 mM NaOAc, $K_d = 132 \pm 10$ μM, $\Delta\%F = 13.2 \pm 0.3$; (■) 50 mM $NaHCO_2$, $K_d = 25.2 \pm 1.3$ μM, $\Delta\%F = 14.3 \pm 0.2$; (▼) 50 mM NaCl, $K_d = 18.8 \pm 0.9$ μM, $\Delta\%F = 17.1 \pm 0.2$; (●) 10 mM $NaNO_3$, 40 mM NaOAc, $K_d = 13.7 \pm 0.7$ μM, $\Delta\%F = 20.6 \pm 0.3$; and (▲) 10 mM $NaNO_2$, 40 mM NaOAc, $K_d = 6.4 \pm 0.7$ μM, $\Delta\%F = 20.9 \pm 0.6$. The data were corrected and the apparent K_d and $\Delta\%F$ calculated as described in the text.

Millner-White and Watts (*20*) determined the abilities of various monovalent anions to promote the formation of a TSAC in the presence of creatine and MgADP. They ranked the effectiveness of anions in the order $NO_2^- > NO_3^- > Cl^- > HCO_2^- > HCO_3^- > Br^- > F^- \gg$ acetate and reported that acetate gives no detectable promotion of TSAC formation. When we titrated CK/80 mM creatine with MgADP in the presence of high concentrations of individual anions (Figure 2), the apparent K_d values for MgADP (Table 2) suggest an identical trend for those anions examined. The values range from 6.4 ± 0.7 μM in the presence of 10 mM NO_2^- to 25.2 ± 1.3 μM in the presence of 50 mM HCO_2^-. Interestingly, $\Delta\%F$ is greatest with NO_2^- and NO_3^- and decreases with the apparent decrease in MgADP affinity.

The change in the intrinsic fluorescence of creatine kinase was followed as the enzyme was titrated with MgADP in the presence of 80 mM creatine and various fixed concentrations of $NaNO_3$. Figure 3 suggests that increasing fixed concentrations of nitrate lead to a decrease in the apparent K_d for MgADP, with a simultaneous increase in the $\Delta\%F$ on saturation of the enzyme with nucleotide. A plot of the apparent K_d for MgADP vs $[NO_3^-]$ (Figure 4) suggests a two-state equilibrium with an apparent K_d for nitrate of 0.39 ± 0.07 mM. A similar set of titrations with MgADP carried

Table 2: Apparent K_d Values for MgADP in the Formation of the CK·MgADP·Anion·Creatine TSAC in the Presence of Saturating Levels of Creatine and Various Anions[a]

anion	K_d, μM	$\Delta\%F$
nitrite	6.4 ± 0.7	20.6 ± 0.6
nitrate	13.7 ± 0.7	20.6 ± 0.3
chloride	18.8 ± 0.9	17.1 ± 0.2
formate	25.2 ± 1.3	14.3 ± 0.2
acetate	132 ± 10	13.2 ± 0.3

[a] Fluorescence quenching titrations were carried out at 30.0 °C in the presence of 50 mM Bicine, 5 mM $Mg(OAc)_2$, pH 8.3, 80 mM creatine, and one of the following: 50 mM NaOAc, 10 mM $NaNO_2$, 10 mM $NaNO_3$, 50 mM NaCl, or 50 mM $NaHCO_2$. The ionic strength was kept constant in the nitrite and nitrate experiments by including 40 mM NaOAc. The experimental protocol and data analysis are the same as shown and described in Figure 1.

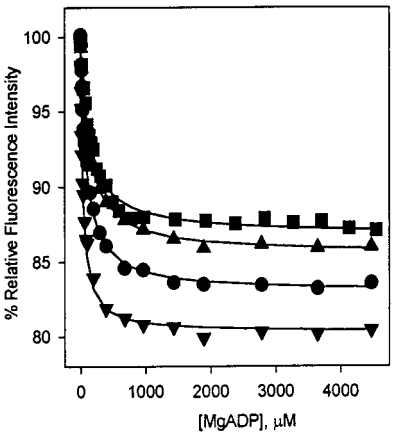

FIGURE 3: Effects of different concentrations of nitrate at saturating levels of creatine on the apparent K_d and $\Delta\%F$ for MgADP binding to creatine kinase as revealed by fluorescence quenching. CK (1 μM) in 50 mM Bicine, 5 mM $Mg(OAc)_2$, 80 mM creatine, and 50 mM NaOAc, pH 8.3, was titrated with MgADP at 30.0 °C in the presence of the following concentrations of $NaNO_3$: (■) none, $K_d = 132 \pm 10$ μM, $\Delta\%F = 13.2 \pm 0.3$; (▲) 0.03 mM, $K_d = 108 \pm 6$ μM, $\Delta\%F = 15.1 \pm 0.2$; (●) 0.20 mM, $K_d = 80.6 \pm 5.3$ μM, $\Delta\%F = 19.6 \pm 0.3$; and (▼) 3.0 mM, $K_d = 32.6 \pm 2.4$ μM, $\Delta\%F = 19.9 \pm 0.3$. The data were corrected and the apparent K_d and $\Delta\%F$ calculated as described in the text.

out in the presence of 10 mM $NaNO_3$ and various fixed concentrations of creatine (Figure 4) suggests that the apparent K_d for creatine from the TSAC is 0.9 ± 0.4 mM.

DISCUSSION

Catalysis by rabbit muscle creatine kinase follows a random-order, rapid-equilibrium mechanism (*27*, *28*). The equilibrium binding data reported herein support our earlier finding from kinetic analysis of the forward reaction (phosphocreatine formation) that recombinant rabbit muscle CK does not show synergism of substrate binding (*15*); i.e., the binding of the first substrate does not enhance the enzyme's affinity of the second substrate. We have used this background to generate the model shown in Figure 5 for the formation of the creatine kinase·MgADP (D)·anion (A)·creatine (C) transition-state analogue complex (TSAC). In this model, D or C may bind first, followed by the other to give the E·D·C complex. The data from Figure 1 confirm that $K_D = K_{CD}$, and thus $K_C = K_{DC}$. The model (Figure 5) also shows that the anion (A) can bind to E·D or E·C, forming the E·D·A or E·A·C ternary complexes, respectively, or to the E·D·C complex to give the E·D·A·C TSAC.

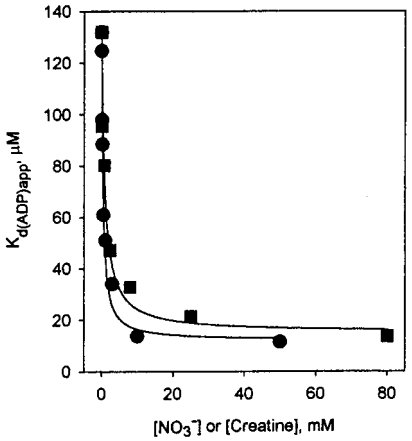

FIGURE 4: Effects of nitrate or creatine and saturating levels of the other on the apparent K_d for MgADP binding to creatine kinase as measured by fluorescence quenching. CK (1 μM) in 50 mM Bicine, 5 mM $Mg(OAc)_2$, and 50 mM NaX (NaOAc + $NaNO_3$), pH 8.3, was titrated with MgADP at 30.0 °C in the presence of the following: (●) 80 mM creatine and the indicated fixed concentrations of $NaNO_3$; (■) 10 mM $NaNO_3$ and the indicated fixed concentrations of creatine. The data were corrected, and the apparent K_d values for the dissociation of either nitrate or creatine were determined as described in the text.

Site-directed mutagenesis has been used to show that a conserved active site tryptophan residue, Trp227 in rabbit muscle CK, is the residue responsible for the intrinsic fluorescence quenching upon the binding of ADP (*8*). The only binding steps that are *directly* observable by fluorescence quenching are the ones involving MgADP binding, i.e., those described by K_D, K_{CD}, and K_{TD} (Figure 5). The data reported in Figure 4 suggest that the apparent K_d for MgADP at saturating levels of creatine decreases with increasing [NO_3^-]. We propose that, at intermediate fixed levels of nitrate, saturation of the enzyme with MgADP in a single titration experiment leads to the formation of two different species, E·D·C and E·D·A·C, which have two different dissociation constants for MgADP, K_{CD} and K_{TD}. The relative amounts of E·D·C and E·D·A·C on saturation of the enzyme are dependent on [NO_3^-], and the data in Figure 4 can be described in terms of the process E·D·A·C $\rightleftharpoons$ E·D·C + A, described by K_{TA} (the dissociation constant for nitrate from the TSAC) = [E·D·C][A]/[E·D·A·C]. The data for different nitrate concentrations were fit to a nonlinear regression analysis as described in Materials and Methods, and it was determined that $K_{TA} = 0.39 \pm 0.07$ mM.

Likewise, at saturating levels of nitrate, the apparent K_d for MgADP decreases with increasing fixed levels of creatine (Figure 4). We propose that, in a single titration experiment at a fixed concentration of creatine, saturation with MgADP again gives two species, E·D·A and E·D·C·A, with different affinities for MgADP. The relative amounts of E·D·A and E·D·C·A on saturation are dependent on the concentration of creatine and reflect the dissociation of creatine from the TSAC, K_{TC}. A value of 0.9 ± 0.4 mM for K_{TC} was determined from nonlinear regression analysis of these data (Figure 4). This represents an approximately 10-fold increase in affinity compared to the K_d and K_M for creatine of 8 mM determined by kinetic analysis (*15*). The apparent K_d for MgADP at saturating levels of both creatine and nitrate represents K_{TD}, the dissociation constant of the nucleotide from the TSAC. At 80 mM creatine and 10 mM nitrate, a

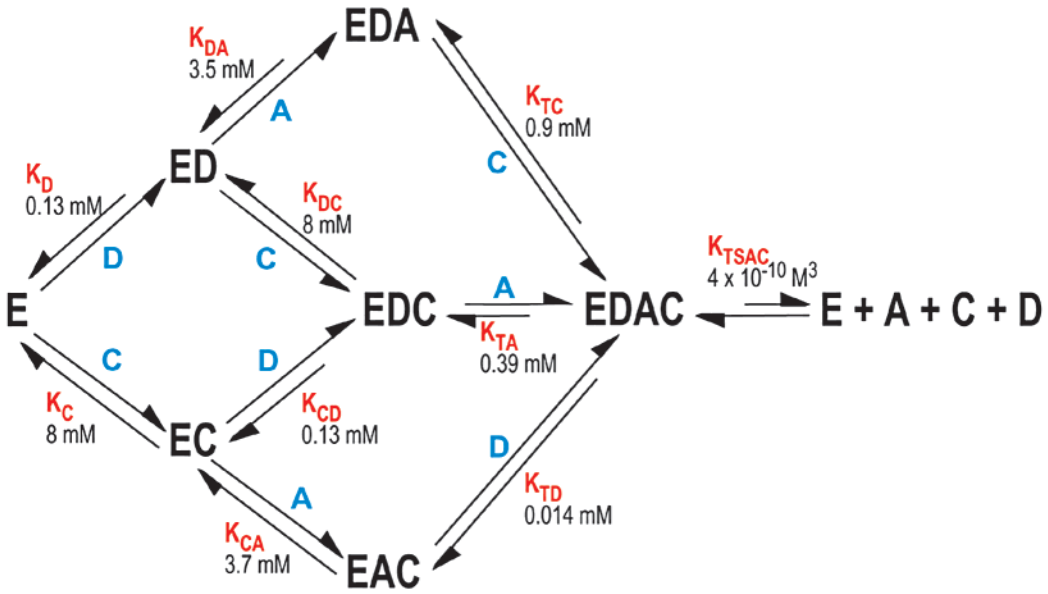

FIGURE 5: A model for the formation of the CK·MgADP·anion·creatine TSAC; E = creatine kinase, D = MgADP, C = creatine, and A = anion = nitrate. See text for details.

value for K_{TD} of 13.7 ± 0.7 μM was obtained. This represents an order of magnitude increase in affinity for the nucleotide compared to the apparent K_d when nitrate is not present (Figure 1).

The use of thermodynamic boxes (Figure 5) allows the estimation of K_{DA} and K_{CA}, the dissociation constants for the dissociation of nitrate from the E·D·A and E·A·C ternary complexes, respectively. This suggests that nitrate binds to either E·D or E·C, but again with a 10-fold lower affinity than for binding to E·D·C to form the E·D·A·C TSAC. The model does not show that nitrate binds to the free enzyme, and in support of this assumption we note that the apparent K_d for MgADP is the same in the absence or presence of 10 mM nitrate (Figure 1).

The experimental procedures reported herein allow the direct determination of not only the apparent K_d for MgADP under various conditions, and indirectly the dissociation constants of creatine and nitrate from the TSAC, but also the $\Delta\%F$ in a given titration experiment. The extent of quenching in a static quenching mechanism is dependent on a number of factors, including, but not restricted to, the distance between the fluorophore and quencher, the orientation of the two, and the polarity of the medium around the fluorophore (*25*). We propose that the reduction of the apparent K_d of MgADP binding to CK in the presence of both creatine and nitrate furnishes additional evidence for the formation of a TSAC (vide supra). We also note a concomitant increase in $\Delta\%F$ when nitrate is used as the anion in formation of the TSAC. This increase of fluorescence quenching could reflect a conformational change of the protein upon forming the TSAC but could also be due in part to fixation of nitrate, a known quencher (*26*), near the active site tryptophan (*18*). We are unable to distinguish between these possibilities at this time but suggest that this increase in $\Delta\%F$ is a second line of evidence for the formation of a TSAC.

Catalysts have been proposed (*29*) to accelerate chemical reactions by preferentially stabilizing the altered substrate in the transition state relative to that of the substrate in the ground state. This principle has been applied to explain the rate enhancements that are brought about by enzymes (*30*). In support of this view, stable analogues that mimic the structural properties of the altered substrate in the transition state are found, in many cases, to be bound by the enzyme with significantly greater affinity than is the substrate itself (*31*−*33*). More recently (*34*), the ability to measure the rates of noncatalyzed reactions has allowed a measure of K_{TX}, the virtual dissociation constant of the altered substrate in the transition state from the enzyme, as exemplified by a value for K_{TX} of 5×10^{-24} M for orotidine-5′-monophosphate decarboxylase (*34*).

The creatine kinase TSAC is intriguing in that it is a quaternary complex, unlike the binary complexes that are the norm with most enzyme−transition-state analogue systems. We used the model shown in Figure 5, the dissociation constants ($K_D = K_{CD} = 0.13$ mM, and $K_A = 0.39$ mM) reported in this paper, and a value of $K_C = 8$ mM [the dissociation constant of creatine from the EC complex as determined by kinetic analysis of the forward reaction (phosphocreatine formation) (*15*)] to determine a value for K_{TDAC}, the constant that describes the dissociation of the enzyme·MgADP·nitrate·creatine TSAC into its individual components. The resulting value of $K_{TDAC} = 3 \times 10^{-10}$ M^3 appears to be the first reported dissociation constant for a quaternary, or even a ternary, TSAC.

From an entropic standpoint, it seems evident that if the elements of MgADP, nitrate, and creatine could be properly combined in a single inhibitor molecule, that inhibitor's tendency to dissociate from the enzyme (with K_d expressed as moles per liter) might be very much lower than the product of three dissociation constants estimated above. Such an inhibitor remains to be synthesized for creatine kinase. Some idea of the potential magnitude of its affinity can be gained, however, from connectivity effects that have been evaluated experimentally for other enzymes, by "cutting" an activated substrate or transition-state analogue inhibitor in two pieces and then comparing the affinities of these pieces with the affinity of the whole molecule. In that way, very high connectivity effects or "effective concentrations", amounting to 10^8 M or more, have been detected in substrates and

transition-state analogue inhibitors of adenosine deaminase, cytidine deaminase, and OMP decarboxylase (*34*). Connectivity effects of this kind seem likely to be especially pronounced in enzymes such as creatine kinase, whose principal function is to bring the substrates together in a configuration appropriate for reaction.

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