Relaxation Spectra of Adenosine Triphosphate–Creatine Phosphotransferase*

Gordon G. Hammes and James K. Hurst†

ABSTRACT: A kinetic study of the reaction catalyzed by creatine kinase has been carried out, primarily using the temperature-jump method. The results obtained indicate that the binding of adenosine di- and triphosphates and their metal complexes with the divalent cations Mg^{2+}, Ca^{2+}, and Mn^{2+} is accompanied by a conformational change of the enzyme–substrate complex. The rates of this process are quite similar in all cases. No conformational changes have been detected with creatine and phosphocreatine binding. An isomerization (or conformational change) of the ternary complex (both nucleotide and creatine substrates bound) may also occur, but a definite interpretation is complicated by relaxation processes associated with metal–nucleotide interactions. Rate constants for the initial association–dissociation of adenosine diphosphate and the metal–adenosine triphosphate complexes with enzyme and the subsequent isomerization are reported, and limits on the corresponding constants for adenosine triphosphate and metal–adenosine triphosphate complexes are placed. At 11° the bimolecular rate constant for adenosine diphosphate association is 2.3×10^7 M^{-1} sec^{-1}, while that for the metal–adenosine diphosphate complex is similar for all three metals, ranging from about 2–7 $\times 10^6$ M^{-1} sec^{-1}. In contrast for most metal–ligand associations, where dissociation of water molecules from the inner coordination sphere of the metal is rate limiting, the bimolecular rate constants associated with Mg^{2+}, Ca^{2+}, and Mn^{2+} vary several orders of magnitude.

Mechanistic implications of the results are discussed. A model consistent with the data is one where the activator cation does not bridge between the enzyme and adenine nucleotide in the enzyme–substrate complex, and discrimination with respect to the metal activator occurs mainly in the rate-determining step.

Creatine kinase (ATP:creatine phosphotransferase, EC 2.7.3.2) catalyzes reaction I. Certain divalent metal

$$\text{ATP} + \text{creatine} \rightleftharpoons \text{ADP} + \text{phosphocreatine} \qquad \text{(I)}$$

ions (Mg^{2+}, Mn^{2+}, and Ca^{2+}) are required for catalytic activity, the true reaction substrates being metal-complexed adenosine phosphates and noncomplexed guanidine base substrates (*cf.* Cleland, 1967). Product inhibition (Morrison and James, 1965) and isotope exchange studies (Morrison and Cleland, 1966; Morrison and White, 1967) have demonstrated the mechanism to be random, rapid equilibrium with phosphate group transfer in the ternary complex rate limiting. Observations of cooperative substrate binding effects (Morrison and James, 1965; Morrison and Uhr, 1966; James and Morrison, 1966; O'Sullivan *et al.*, 1966; Jacobs and Cunningham, 1968) and alterations in the presence of substrates of chemical reactivities with irreversible inhibitors of sulfhydryl (O'Sullivan *et al.*, 1966; O'Sullivan and Cohn, 1966b) and other groups (Watts, 1963; Lui and Cunningham, 1966), in solvent proton nuclear magnetic resonance relaxation rates in the presence of paramagnetic ions (Mn^{2+}) (O'Sullivan and Cohn, 1966b) and in a variety of other physicochemical parameters of the protein (Lui and Cunningham, 1966; Samuels *et al.*, 1961) have been interpreted as indicating enzyme conformational changes accompany substrate binding.

In the present study rapid kinetic techniques have been used to investigate intermediates in the reaction pathway. The results obtained indicate that binding of adenosine phosphates and their metal complexes (Mg^{2+}, Mn^{2+}, and Ca^{2+}) induces a conformational change in the protein. The isomerization rate is independent of the presence of activator ion, and no isomerization was observed in the presence of creatine substrates alone. A second, slower isomerization in the system at equilibrium, *i.e.*, in the presence of all substrates, was suggested, but unambiguous interpretation of the data proved impossible due to interfering metal–nucleotide interactions. Rate constants for the initial association and dissociation of ADP and MADP complexes with the enzyme have also been determined. The rate constants associated with these reactions are virtually identical for the three metal ions investigated, quite unlike normal metal–ligand substitution reactions (Eigen and Wilkens, 1964). This finding is in agreement with the contention that the metal ion does not bridge substrate and enzyme (Cohn and Leigh, 1962; O'Sullivan and Cohn, 1966a).

* From the Department of Chemistry, Cornell University, Ithaca, New York 14850. *Received October 9, 1968.* This work was supported by a grant from the National Institutes of Health (GM 13292).

† National Institutes of Health Postdoctoral Fellow, 1966–1968.

Experimental Section

Materials

Creatine kinase from rabbit muscle was obtained according to procedure B of Kuby *et al.* (1954) and recrystallized according to the modifications of Mahowald *et al.* (1962). The twice-recrystallized enzyme was stored at −10° as crystals in slightly ammoniacal solution for periods up to 6 months without noticeable decomposition. One preparation assayed by following the rate of phosphocreatine production (Kuby *et al.*, 1954) gave a specific activity of 56 Kuby units, which corresponded to an activity of 120 μequiv/(mg min) when the rate of proton release was followed (Mahowald *et al.*, 1962). The pH-Stat method was employed for routine assays; various enzyme preparations gave specific activities corresponding to 51–56 Kuby units. A Radiometer Type TTTl Titrator and Titrigraph assembly was used for these assays. On several occasions marked curvature in the initial velocity slopes, suggestive of enzyme denaturation, was noticed. Prior incubation of the pH electrode with dilute (10^{-3} M) 2-mercaptoethanol removed this difficulty, and subsequent initial velocity slopes were linear. The enzyme preparations were tested for homogeneity by column chromatography on DEAE-Sephadex and disc electrophoresis on polyacrylamide gels. Elution from DEAE-Sephadex A-50 columns (50 cm, 50-ml bed volume) with a Tris buffer gradient (0.02–0.1 M, pH 8.0) gave single broad bands; after dialysis overnight against 1.6×10^{-3} M NH_4OH, various fractions of the band gave identical specific activities of 46–56 Kuby units. Electrophoresis showed only a single major band accompanied by one or two faster moving bands present in trace amounts.

Nucleotides were obtained from P-L Biochemicals. Paper chromatograms, using the descending front method and a 0.15 M sodium citrate (pH 4.0)–ethanol–1-butanol (6:10:1, v/v) solvent system (F. G. Walz, private communication), indicated only traces of nucleotide impurities were present. Creatine was obtained from Eastman and creatine phosphate from Calbiochem. Other chemicals were reagent grade and used without further purification.

Kinetic Studies. A temperature-jump apparatus, previously described (*cf.* French and Hammes, 1969), was the primary kinetic tool employed in these studies. With this apparatus relaxation times from 50 μsec to 100 msec could be conveniently measured. A 30-kV pulse passed through 0.1 M KCl solutions gave a temperature rise of *ca.* 8° in less than 20 μsec. Reagent solutions were prepared just before use. Adenosine phosphate and enzyme concentrations were determined spectrophotometrically: for nucleotides, ϵ_{259} 1.54×10^4 cm^{-1} M^{-1} at pH 7, while for creatine kinase, ϵ_{280} 7.2×10^4 cm^{-1} M^{-1} at pH 7.0 (Kuby and Noltmann, 1962). For all temperature-jump experiments the pH of reaction solutions was adjusted to 7.6 at approximately 25°, solutions were thermostated at 3°, the equilibrium temperature after perturbation being *ca.* 11°, and the ionic strength was maintained at 0.1 M with KCl. The approach to equilibrium after perturbation was monitored at 558 mμ with phenol red, concentration of the indicator dye in solution generally being 2×10^{-5} M. Thus, only those effects producing a pH change could be observed. Amplitudes of relaxations measured comprised 0.1–2% of the total signal. Current discharges through the reactant solutions appeared to gradually denature the enzyme. After repeated jumps precipitation occurred and enzyme solutions assayed after perturbation suggested an inactivation rate of *ca.* 1%/temperature jump. To minimize the error introduced by this inactivation reactant solutions were changed frequently (about every 15–20 jumps). Reaction solutions were routinely assayed at the end of temperature-jump experiments to ensure that the enzyme had good activity.

Stopped-flow measurements were made with a Gibson-Durrum apparatus equipped with a Kel-F syringe block and mixing chamber.

Thermodynamic Constants. Analysis of the relaxation data obtained requires knowledge of metal–nucleotide and enzyme–substrate binding constants. Their selection from available data in the literature is not always a trivial matter. Reported apparent binding constants are often quite sensitive to the nature and concentration of buffering components as well as temperature, pH, and ionic strength. In this case, such considerations are especially relevant to the metal–nucleotide stability constants (*cf.* O'Sullivan and Perrin, 1964).

Apparent stability constants at pH 7.6 for metal–adenosine di- and triphosphates were calculated from the data of Khan and Martell (1966, 1967). The constants were corrected to 11° with ΔH° values for MATP complexes of −6 kcal/mole and for MADP of −5 kcal/mole (Burton, 1959). (The ΔH° values for calcium and manganese adenosine phosphates were assumed to be the same as those reported by Burton (1959) for the corresponding magnesium complexes.) Thus calculated, the apparent stability constants at pH 7.6 are: for ADP, Mg^{2+}, $K_1 = 9.1 \times 10^2$ M^{-1}; Ca^{2+}, $K_1 = 4.55 \times 10^2$ M^{-1}; Mn^{2+}, $K_1 = 9.1 \times 10^3$ M^{-1}; and for ATP, Mg^{2+}, $K_1 = 9.1 \times 10^3$ M^{-1}; Ca^{2+}, $K_1 = 5.3 \times 10^3$ M^{-1}; Mn^{2+}, $K_1 = 3.3 \times 10^4$ M^{-1}, where $K_1 = \Sigma(ML_i)/(M)(\Sigma L_i)$, M is the metal ion and L_i is a particular ligand species.

In contrast to the apparent constants for metal–concleotide association, those reported for ADP binding to creatine kinase show very little systematic variation with temperature, pH, and medium changes. The dissociation constants selected are $K_B = (E)(ADP)/\Sigma(EADP)_i = 1.0 \times 10^{-4}$ M (Kuby *et al.*, 1962). $K_{MB} = (E)(MADP)/\Sigma(EMADP)_i$; for Mg^{2+},[1] $K_{MB} = 1.2 \times 10^{-4}$ M; for Ca^{2+}, $K_{MB} = 9 \times 10^{-5}$ M; for Mn^{2+},[1] $K_{MB} = 7 \times 10^{-5}$ M (Morrison and Uhr, 1966). $K_B' = (E)(ATP)/\Sigma(EATP)_i = 4 \times 10^{-4}$ M (Kuby *et al.*, 1962). $K_{eq} = (MgADP)(phosphocreatine)/(MgATP)(creatine) = 1.1 \times 10^{-2}$ (Kuby and Noltmann, 1962). The value of K_{eq} was assumed to be identical for all three metal ions; as will be evident this is an adequate approximation for our purposes. For those cases where simultaneous equilibria were present in reaction solutions concentrations of reactant species were obtained by iterative solution. Successive iterations were made until calculated concentrations agreed to within about 1%.

[1] Average of constants reported by Morrison and Uhr (1966) and O'Sullivan and Cohn (1966a).

Results and Treatment of Data

Creatine Kinase in the Absence of Substrates. An enzyme solution (*ca.* 8×10^{-5} M)[2] containing also 2×10^{-5} M phenol red and 0.1 M KCl, showed no relaxation phenomena over the entire range of accessible time scales. The effect of different indicator dye concentrations upon a system containing in addition (ATP) = 1.5 mM (for which a relaxation effect was observed with $\tau = 60$ μsec) was investigated. A ninefold variation in phenol red concentration produced no change in the measured relaxation time, concentrations of enzyme and nucleotide being maintained constant (Table IV, 7–11). These results obviate considerations of enzyme–dye interactions.

Solutions containing enzyme and metal ions (Mg^{2+}, Mn^{2+}, or Ca^{2+}) in the concentration ranges spanning those used for the investigation of enzyme interactions with metal–nucleotide complexes ((E) = $0.2–1.2 \times 10^{-4}$ M, (M) = 0.1–10 mM) likewise showed no definite relaxation effects in the measured time range. Short relaxation times ($\tau \simeq 40$ μsec) may have been seen in the enzyme–metal ion systems at the lowest reactant concentrations, but these effects were of very low amplitude and generally not reproducible. No relaxation effects were seen in solutions containing just phenol red or phenol red and metal ions.

Creatine Kinase and Guanidine Base Substrates. No relaxation processes were observed in solutions of enzyme and creatine in the concentration ranges $(E)_{total} = 6–8 \times 10^{-5}$ M, $(creatine)_{total} = 0.5–21$ mM, nor were any seen in solutions of enzyme, $(E)_{total} = 8–9 \times 10^{-5}$ M, and phosphocreatine, $(phosphocreatine)_{total} = 0.9–47$ mM.

Adenosine Phosphates and Metal–Adenosine Phosphate Complexes. Reaction solutions containing ADP or ATP at low concentrations (0.1–0.6 mM) showed fast relaxation effects ($\tau = 30–90$ μsec). The reciprocal relaxation times were a linear function of total nucleotide concentration, suggesting a proton transfer process (Diebler *et al.*, 1960). Magnitudes of relaxation times are comparable to those previously reported for proton transfer in these systems under slightly different conditions (Diebler *et al.*, 1960; Eigen and Hammes, 1961). The equilibrium shift accompanying the temperature jump was toward more alkaline conditions, opposite to the change associated with the enzyme–substrate interactions described below. In solutions of creatine kinase containing nucleotides at low concentrations no interference from these proton transfer reactions could be detected; first-order plots of the equilibrium shift toward more acidic conditions were strictly linear. Presumably, the presence of the enzyme increased the buffer capacity of the reactant solutions sufficiently so that these proton transfer reactions became too rapid to observe. In solutions of adenosine phosphates alone, no relaxation processes were detected at higher nucleotide concentrations.

In general, solutions containing nucleotides at low concentrations and metal ions did not exhibit relaxation phenomena,[3] while at higher nucleotide concentrations (0.5–10 mM) relaxation effects were seen in the millisecond range, more than one often being seen at the highest nucleotide concentrations. No detailed analysis of the relaxation spectra was attempted; the phenomena are tentatively ascribed to polynuclear association–dissociation equilibria and/or protolytic reactions between polynuclear species.

Relaxation data relevant to considerations of the enzyme–substrate interactions reported below are collected in Table I. Also included are relaxation times measured for solutions containing approximate equilibrium concentrations of substrates, enzyme being absent. These relaxation times are roughly those expected from the corresponding metal–ADP and metal–ATP systems. In general,[3] relaxation times ascribed to creatine kinase isomerizations in the presence of nucleotide substrates reported below are considerably faster ($\tau = 50–100$ μsec) than these so that the metal–nucleotide interactions can be expected not to interfere in the analysis of enzyme–nucleotide interaction data. Similarly, the relaxation times attributed to the bimolecular associations of ADP and MADP with creatine kinase ($\tau = 200–1000$ μsec) occur only at very low ADP concentrations, under conditions for which none of the relaxation processes discussed above appear. Again, amplitudes of the relaxation processes described in Table I were markedly diminished in the presence of enzyme.

Creatine Kinase and ADP. A single rapid relaxation process ($\tau = 60–150$ μsec) occurred when solutions containing creatine kinase and ADP were perturbed. The effect was studied over a range of 4-fold variation in enzyme and 100-fold variation of ADP concentrations. The data are summarized in Figure 1. The relaxation time decreased rapidly with increasing concentrations of reactants at low reactant concentrations, but became independent of concentration at high ADP levels, which is characteristic of a reaction mechanism of the type (Amdur and Hammes, 1966).

$$E + ADP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (EADP)_1 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (EADP)_2 \qquad (1)$$

The general solution of this coupled system requires solution of a quadratic determinant, but in the limit where the first step equilibrates rapidly relative to the second, the relaxation time for the slower process can be written as (Amdur and Hammes, 1966)

[2] Creatine kinase contains two active sites per protein molecule. In this paper enzyme concentrations will be expressed in terms of numbers of active sites, *i.e.*, twice the molar concentrations.

[3] An exceptional case was the magnesium–ADP system, for which a fast relaxation process ($\tau \simeq 40$ μsec) occurred at nucleotide concentrations of (ADP) $\simeq 2$ mM. Similar effects in solutions containing Mg^{2+} and ADP have been ascribed to metal–ligand association equilibria (Eigen and Hammes, 1961). Calculation of the relaxation time expected for metal–ligand associations under the present conditions from reported rate constants (Diebler *et al.*, 1960; Eigen and Hammes, 1961) yielded a value of $\tau = 30$ μsec, in good agreement with the measured relaxation time. No fast relaxation processes were observed for magnesium–ATP solutions under these conditions, which is consistent with published reports (Eigen and Hammes, 1961).

TABLE I: Relaxation Processes in Metal–Nucleotide Solutions at 11°.

System	(Metal)[a] (mM)	(Nucleotide)[a] (mM)	τ (msec)
Mg + ADP + phenol red	11	0.18–1.2	
	9.7	2.5	0.04
	9.4	3.4	10–11
Ca + ADP + phenol red	9.1	0.14	
	8.7	0.7–1.6	63
	8.5	3.5	0.5, 5.2, ~50
Mn + ADP + phenol red	0.2–1.2	0.2–1.0	15–30
	9.7	1.8	1.3, 94
Mg + ATP + phenol red	1.0	0.2–0.4	
	1–3.2	0.6–3.2	8–16
Ca + ATP + phenol red	11	0.25	
	10–11	0.7–1.4	~50
	10	3.7	8
Mn + ATP + phenol red	1.2	0.23	
	1.2	0.47–1.3	60–80
Mg + ADP + ATP + creatine + phosphocreatine + phenol red[b]	8.2	4.0	0.12, 7.9
Ca + ADP + ATP + creatine + phosphocreatine + phenol red[b]	8.4	4.0	6.5
Mn + ADP + ATP + creatine + phosphocreatine + phenol red[b]	4.7	4.0	3.0, 92

[a] Refers to total concentration of species in solution. [b] "Equilibrium" concentrations: (ADP) = 2.3 mM, (ATP = 1.7 mM, (creatine) = 37 mM, (phosphocreatine) = 0.3 mM, expressed as total concentration of species in solution

$$1/\tau_2 = k_{-2} + k_2/[1 + K_a/((\bar{E}) + (\overline{ADP}))] \quad (2)$$

where $K_a = k_{-1}/k_1 = (E)(ADP)/(EADP)_1$, is the equilibrium constant for the initial substrate association and the symbols $(\bar{E})$ and $(\overline{ADP})$ refer to equilibrium concentrations of the nonassociated species. The equation can be rearranged to the form

$$[1/\tau_2 - k_{-2}]^{-1} = (1/k_2)[1 + K_a/((\bar{E}) + (\overline{ADP}))]$$

Various values of k_{-2} can be assumed in a trial-and-error process until a linear fit of the data is obtained, and k_2 and K_a can be determined from the values of the slope and intercept.

Application of this method to the data gave a good straight line with $k_{-2} = 2.4 \times 10^3$ sec^{-1} as shown in Figure 2. The calculated over-all binding constant, $K_B = (E)(ADP)/[(EADP)_1 + (EADP)_2] = K_a/(1 + K_b)$, $K_b = k_2/k_{-2}$, agrees favorably with the thermodynamically measured constant, $K_B = 1.0 \times 10^{-4}$ M. The parameters obtained are as follows: $k_2 = 1.67 \pm 0.35 \times 10^4$ sec^{-1}, $k_{-2} = 2.4 \times 10^3$ sec^{-1}, $K_a = 6.0 \pm 1.5 \times 10^{-4}$ M and $K_B = 7.5 \pm 3 \times 10^{-5}$ M. Error limits were established from standard deviations of the slope and intercept. For $k_{-2} \geq 2.7 \times 10^3$ sec^{-1}, curvature of the plot in Figure 2 was noticeable, while for $k_{-2} \leq 2.0 \times 10^3$ sec^{-1}, the calculated binding constant differs considerably from the thermodynamic constant. Therefore, the error in k_{-2} can be roughly assessed as ±20%. The theoretical curve for the above mechanism calculated from the determined kinetic constants using eq 2 is plotted as a solid line in Figure 1. The rate constants k_1 and k_{-1} have been evaluated from the analysis of relaxation effects in solutions containing metal ions as discussed below. The theoretical curve obtained for the coupled system, *i.e.*, from solution of the quadratic determinant (*cf.* Amdur and Hammes, 1966), is given in Figure 1 as the dashed line. The reasonable agreement between theoretical curves attests to the validity of the approximation made in this analysis.

Creatine Kinase, ADP, and Activator Ions (Mg^{2+}, Ca^{2+}, or Mn^{2+}). In solutions containing enzyme, metal ions and ADP at concentrations above ~10^{-4} M only single relaxation processes ($\tau_2 = 60$ μsec), independent of reactant concentrations over a 100-fold range, are observed. At ADP levels below 10^{-4} M, a second relaxation process appears ($\tau_1 = 200$–1200 μsec); the relaxation times associated with this process increased rapidly with increasing ADP concentration. The data are consistent with the general reaction scheme.

$$\begin{array}{ccccc} & M & & M & & M \\ & + & & + & & + \\ E + & ADP & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & (EADP)_1 & \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} & (EADP)_2 \\ & \| K_1 & & \| K_2 & & \| K_3 \\ E + & MADP & \underset{k_{-1}'}{\overset{k_1'}{\rightleftharpoons}} & (EMADP)_1 & \underset{k_{-2}'}{\overset{k_2'}{\rightleftharpoons}} & (EMADP)_2 \end{array} \quad (3)$$

This mechanism is characterized by five relaxation times.

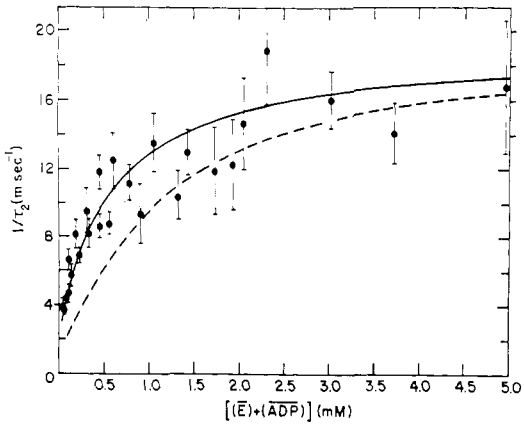

FIGURE 1: The reciprocal relaxation time for creatine kinase–ADP interactions $1/\tau_2$, as a function of the sum of the equilibrium concentrations of enzyme and ADP. Each data point is the average of four to eight determinations; error limits are the standard deviations of the determinations. The solid curve represents the theoretical line for the uncoupled system calculated from the kinetic constants determined as in Figure 2. The dashed curve is the corresponding theoretical line for the coupled system.

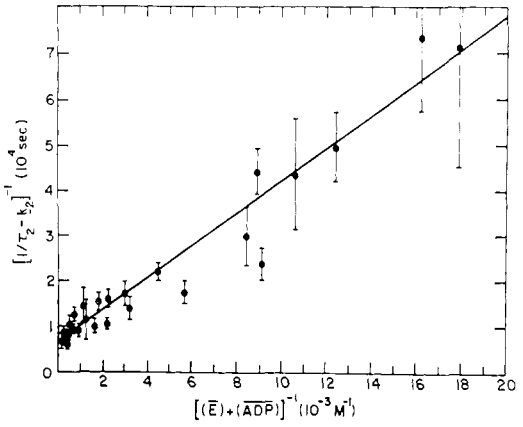

FIGURE 2: A plot of $[(1/\tau_2) - k_{-2}]^{-1}$ *vs.* $[(E) + (ADP)]^{-1}$ with $k_{-2} = 2.4 \times 10^3$ sec^{-1}. Data points are the same as those plotted in Figure 1; the line was determined by a least-squares analysis of the data.

The metal–ligand associations described by the vertical steps can reasonably be expected to equilibrate rapidly relative to the horizontal steps when Mn^{2+} or Ca^{2+} are the metal ions present (Eigen and Hammes, 1961; Eigen and Wilkins, 1964). Hence, three relaxation processes corresponding to the uncoupled metal–ligand association–dissociation equilibria are too rapid to measure and only two relaxation times are expected, which is in accord with the experimental findings. The assumption of rapidly equilibrating vertical steps is also made for systems containing Mg^{2+}, although here the approximation is less valid. Determination of individual rate constants from the measured relaxation times requires solution of a quadratic determinant of the form

$$\begin{vmatrix} a_{11} - (1/\tau) & a_{12} \\ a_{21} & a_{22} - 1/\tau \end{vmatrix} = 0$$

for which

$$a_{11} = k_1^*[(\overline{MADP}) + (\bar{E})/(1 + 1/K_1(M))] + k_{-1}^*/[1 + 1/K_2(M)]$$
$$a_{12} = k_{-1}^*/[1 + 1/K_2(M)]$$
$$a_{21} = k_2^*/[1 + 1/K_2(M)]$$
$$a_{22} = k_2^*/[1 + 1/K_2(M)] + k_{-2}^*/[1 + 1/K_3(M)]$$

where $k_1^* = k_1' + k_1/K_1(M)$, $k_{-1}^* = k_{-1}' + k_{-1}/K_2(M)$, $k_2^* = k_2' + k_2/K_2(M)$, and $k_{-2}^* = k_{-2}' + k_{-2}/K_3(M)$. (The derivation of these relationships is given in the Appendix.)

Two limiting cases are that the isomerization of the enzyme–substrate complex is either very fast or very slow relative to the bimolecular association step. In the former case, the second step is at all times essentially at equilibrium relative to the first, or $a_{22} - (1/\tau_2) = 0$ (*cf.* Castellan, 1963), *i.e.*, $1/\tau_2 = k_2^*/[1 + 1/K_2(M)] + k_{-2}^*/[1 + 1/K_3(M)]$ and $1/\tau_1 << 1/\tau_2$. In the latter case, $a_{11} - (1/\tau_1) = 0$ and $1/\tau_2 = a_{22} - a_{12}a_{21}/a_{11}$. Substituting the expressions for the a_{ij}'s gives

$$1/\tau_2 = k_{-2}^*/[1 + 1/K_3(M)] + \frac{k_2^*/[1 + 1/K_2(M)]}{1 + \dfrac{k_{-1}^*/[1 + 1/K_2(M)]}{k_1^*\,((\bar{E}) + (\overline{MADP}))}}$$
$$1/\tau_1 >> 1/\tau_2$$

At very high levels of $(\bar{E}) + (\overline{MADP})$, *e.g.*, approaching saturation of the enzyme binding sites, this equation reduces to

$$1/\tau_2 = k_2^*/[1 + 1/K_2(M)] + k_{-2}^*/[1 + 1/K_3(M)]$$

which is identical with that obtained for the other limiting case. The equations predict that in these limits $1/\tau_2$ should be a function of the concentrations of noncomplexed metal ion present. In the absence of metal ion, the relationships reduce to $1/\tau_2 = k_2 + k_{-2}$, while at high metal concentrations ($K_2(M)$, $K_3(M) >> 1$) $1/\tau_2 = k_2' + k_{-2}'$, as required by the mechanism. The reciprocal of relaxation times determined under conditions approaching both of the above limiting cases for solutions of enzyme, ADP and each of the three metal ions are presented in Figure 3 as a function of metal ion concentration. Experiments in systems containing enzyme and ATP, with metal ion concentrations varied over a much wider range, had indicated that at very high concentrations of Ca^{2+} and Mn^{2+} the relaxation effect ($\tau_2 = 50$ μsec) observed at lower metal ion concentrations disappeared. Such difficulties were not encountered with Mg^{2+} solutions. Relevant data are listed in Table V (compare 8–10, 14–17, 23–27). To minimize the possibility of side-effect interferences in the corresponding ADP systems, excessive metal ion concentrations were avoided. Such considerations place a relatively low upper limit on the metal ion concentration range that can be investigated.

Within experimental error, the isomerization relaxation time is independent both of the concentration and nature of the activator metal ion present. The simplest

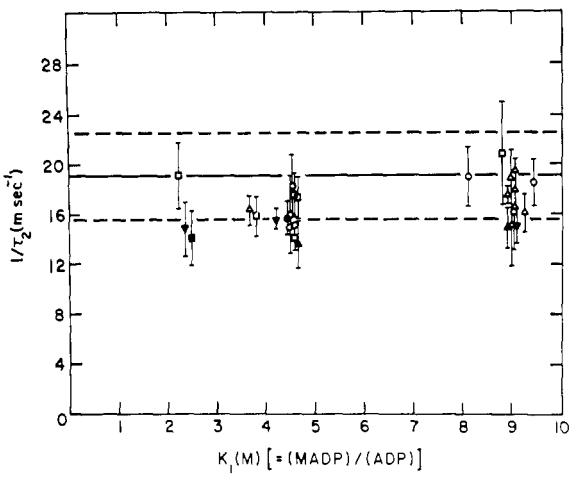

FIGURE 3: Reciprocal relaxation times for isomerization in solutions containing creatine kinase, ADP, and divalent metal ions as a function of K_i (M). For all points the experimental conditions, $1/\tau_1 \ll 1/\tau_2$ or $1/\tau_1 \gg 1/\tau_2$, are approached. Each point is the average of five to eight determinations; the error limits are standard deviations of the determinations. Symbols used are: ○ = Mg, □ = Ca, △ = Mn; filled symbols indicate the presence of creatine, (creatine) = 36 mM. ▼ refers to measurements of the system at equilibrium: (ADP) ≃ 2.3 mM, (ATP) ≃ 1.7 mM, (creatine) ≃ 36 mM, (phosphocreatine) ≃ 0.3–0.4 mM. The solid line is $k_2 + k_{-2}$, with the dashed lines representing error limits for that sum.

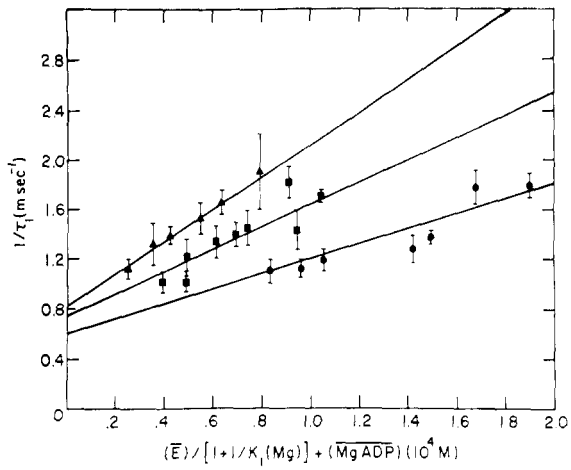

FIGURE 4: Reciprocal relaxation time for the association of ADP and MgADP with creatine kinase as a function of enzyme and MgADP concentrations at various concentrations of Mg^{2+}; ●, (Mg) = 4.95 mM; ■, (Mg) = 2.49 mM; ▲, (Mg) = 1.35 mM. Each data point is the average of five to eight determinations; error limits are the standard deviations in the determinations. The curves are the theoretical lines for the coupled solution with k_1* taken as the average value calculated from the individual points at each concentration of Mg.

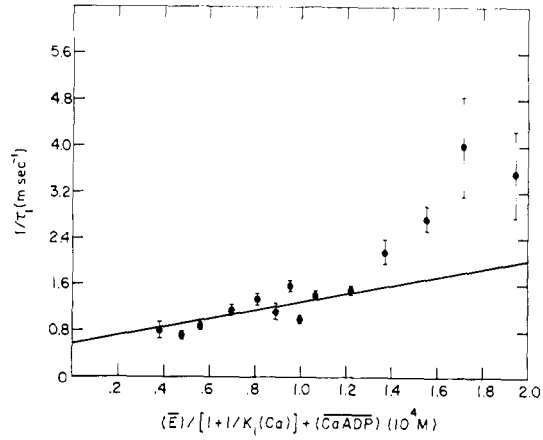

FIGURE 5: Reciprocal relaxation time for the association of ADP and CaADP with creatine kinase as a function of enzyme and CaADP concentrations, (Ca) = 9.82 mM. Data points are the average of five to eight determinations and the error limits are the standard deviations. The solid curve is calculated for the coupled solution with k_1* taken as the average of values calculated from individual points, excluding those points for which $1/\tau_1 > 2.0$ msec^{-1}.

explanation of these results is that $k_2 = k_2'$ and $k_{-2} = k_{-2}'$, for which $K_2 = K_3$ by the principle of detailed balancing and the expressions for $1/\tau_2$ reduces to $1/\tau_2 = k_2 + k_{-2}$, independent of metal ion. The alternate possibility of fortuitous combinations of rate and binding constants in different metal ion systems leading to an apparent independence of metal ion under the experimental conditions seems unlikely. The presence of creatine in concentrations which bind 70–80% of the corresponding substrate sites on the enzyme[4] ((creatine) = 3.5×10^{-2} M), also has little effect upon the isomerization kinetics (Figure 3); data are presented for both the dead-end E-MADP-creatine complexes and the equilibrium systems.

The result that $k_2 = k_2'$, $k_{-2} = k_{-2}'$ for all activator ions greatly simplifies the task of solving the quadratic determinant. The relationships, $K_2 = K_3 = K_1K_B/K_{MB}$ and $k_{-1}^* = k_1^*(1 + K_b)K_{MB}$, hold (see the Appendix) and the elements of the determinant reduce to

$$a_{11} = k_1^*[(\overline{MADP}) + (\bar{E})/(1 + 1/K_i(M))] + (1 + K_b)K_{MB}/[1 + 1/K_2(M)]$$

$$a_{12} = k_1^*(1 + K_b)K_{MB}/[1 + 1/K_2(M)]$$

$$a_{21} = k_2$$

$$a_{22} = k_2 + k_{-2}$$

Solution for k_1* from experimentally determined relaxation times is then trivial since the concentrations of reactant species and all other constants are known.

The relaxation time, τ_1, corresponding to the association of enzyme and nucleotide was measured over the experimentally accessible range at various constant concentration levels of metal ion. The lower concentration limit was dictated by instrument resolution, relaxation amplitudes being too small to detect below reactant concentrations, $(\bar{E}) + (\overline{MADP})$, of 4×10^{-5} M, while the

[4] Creatine binding is markedly dependent upon the presence of nucleotide substrates (*cf.* O'Sullivan *et al.*, 1966; Jacobs and Cunningham, 1968). Concentration levels of creatine present in these experiments are sufficient to cause binding at most of the creatine-binding sites when metal–nucleotides are also bound, but binding would be very incomplete (*ca.* 20–30% saturation) in the absence of the second substrate.

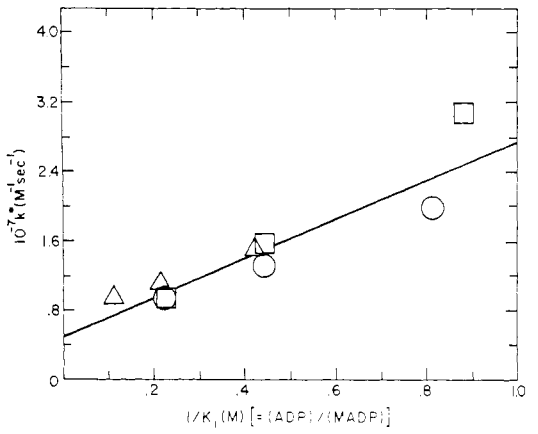

FIGURE 6: The effective association rate constant, k_1^*, as a function of $1/K_1(M)$ for the three divalent cations; ○ = Mg, □ = Ca, △ = Mn. The line determined by the methods of least squares gives $k_1' = 5.1 \times 10^6$ M^{-1} sec^{-1} and $k_1 = 2.2 \times 10^7$ M^{-1} sec^{-1}.

upper limit occurred at reactant concentrations giving rise to $\tau_2 < 200$ μsec; at higher reactant concentrations resolution of the two relaxation times, τ_1 and τ_2, could not be accurately obtained. The rate constants, k_1^*, were calculated from individual data points. The data obtained for creatine kinase, ADP, and Mg^{2+} at several concentrations of metal ion are presented in Figure 4. The average values of k_1^* were taken to construct theoretical curves describing the concentration dependence of $1/\tau_1$ for the systems.

For systems containing Mn^{2+} or Ca^{2+}, rate–concentration plots deviated significantly from the corresponding theoretical curves at high reactant concentrations. The upward deflection became pronounced for relaxation times less than 500 μsec and these points were not included in the subsequent analysis of the data. An example of the curvature for a reaction solution containing Ca^{2+} is given in Figure 5. Under other conditions curvature was even more pronounced. No explanation of this effect can be found within the confines of the proposed mechanism. (The deviations could not be accounted for by coupling the step, M + EADP$_i$ ⇌ MEADP$_i$, to the system, *i.e.*, by not assuming that vertical steps equilibrate rapidly.) Most likely, the explanation will lie in the consideration of polynuclear metal–nucleotide interactions and further discussion is postponed until additional understanding of these effects is reached. The justification for ignoring this complicating factor is the excellent internal consistency exhibited in the remaining data. Values of k_1^* for the different metals are summarized in Table II.

Secondary plots of k_1^* against $1/K_1(M)$ permit the evaluation of the rate constants, k_1 and k_1'. Dissociation rate constants can be calculated from the equilibrium relationships $k_{-1} = K_B(1 + K_b)k_1$ and $k_{-1}' = K_{MB}(1 + K_b)k_1'$. The rate constants obtained are listed in Table III. The requirement that the constants k_1 and k_{-1} be identical in the three different metal ion systems is adequately met. The most striking result is the virtual independence of k_1' and k_{-1}' upon the particular diva-

TABLE II: Effective Bimolecular Rate Constants for the Interaction of Creatine Kinase with ADP–MADP at 11°.

System	(M) (mM)	$10^{-7}\ k_1^*$[a] (M^{-1} sec^{-1})
E–Mg–ADP–phenol red	1.35	2.0 ± 0.05
	2.5	1.3 ± 0.2
	4.95	0.94 ± 0.09
E–Ca–ADP–phenol red	2.5	3.1 ± 0.4
	4.9	1.6 ± 0.3
	9.8	0.94 ± 0.2
E–Mn–ADP–phenol red	0.26	1.5 ± 0.6
	0.52	1.1 ± 0.7
	1.0	0.95 ± 0.16

[a] Error limits represent one standard deviation for k_1^* calculated from individual data points.

TABLE III: Rate Constants for Binding of ADP and Metal–ADP Complexes to Creatine Kinase at 11°[a].

Metal Ion	$10^{-7}k_1$ (M^{-1} sec^{-1})	$10^{-4}k_{-1}$ (sec^{-1})	$10^{-7}k_1'$ (M^{-1} sec^{-1})	$10^{-4}k_{-1}'$ (sec^{-1})
Mg^{2+}	1.8	1.4	0.53	0.51
Ca^{2+}	3.3	2.6	0.17	0.12
Mn^{2+}	1.8	1.5	0.74	0.41

[a] Rate constants are estimated to be accurate to within a factor of 2–3.

TABLE IV: Relaxation Effects in Solutions of Creatine Kinase and ATP at 11°.

No.	($\bar{E}$ + $\overline{ATP}$) (mM)	$1/\tau$ (msec^{-1})[a]
1	0.066	13.9 ± 0.9
2	0.098	9.4 ± 1.5
3	0.32	17.8 ± 1.3
4	0.54	21.3 ± 0.8
5	0.94	15.5 ± 1.5
6	1.1	15.3 ± 3.1
7	1.5, (phenol red) = 0.95×10^{-5}	20.0 ± 2.3
8	1.5, (phenol red) = 2.0×10^{-5}	16.4 ± 0.9
9	1.4, (phenol red) = 4.1×10^{-5}	16.7 ± 0.9
10	1.4, (phenol red) = 6.8×10^{-5}	17.0 ± 2.5
11	1.4, (phenol red) = 8.2×10^{-5}	17.4 ± 1.4
12	1.6	14.2 ± 0.2
13	1.7	15.9 ± 2.8
14	2.3	22.0 ± 2.6
15	2.9	16.4 ± 2.6

[a] Average of four to eight determinations; the error limits designate the standard deviation of the determinations.

TABLE V: Relaxation Effects in Solutions of Creatine Kinase, ATP, and Metal Ions at 11°.

No.	10^4(ATP)[a] (M)	10^4(E)[a] (M)	(M) (mM)	K_1(M) $\left(=\frac{(MATP)}{(ATP)}\right)$	$1/\tau$[b] ($msec^{-1}$)
			Mg		
1	0.48	0.99	0.98	9.0	21 ± 3
2	0.72	0.99	0.96	8.8	21 ± 2
3	1.1	1.1	0.97	8.8	14 ± 2
4	1.6	0.98	1.0	9.1	20 ± 5
5	3.6	1.1	0.99	9.0	16 ± 2
6	7.4	1.1	0.99	9.0	17 ± 2
7	15	1.0	0.98	8.9	17 ± 2
8	28	0.88	0.95	8.6	17 ± 2
9	8.8	0.65	4.2	38	20 ± 3
10	8.6	0.67	17	156	21.5 ± 1
			Ca		
11	1.0	1.1	2.0	10.5	17 ± 2
12	3.2	1.1	2.0	10.5	17 ± 2
13	6.9	1.1	2.1	11	17 ± 3
14	14.4	1.0	2.0	10.5	15 ± 1
15	10	0.95	4.3	22	23 ± 2
16	9.9	0.93	14	73	[c]
17	9.6	0.90	28	147	[c]
			Mn		
18	0.35	0.35	1.0	33	14 ± 1
19	0.72	0.35	0.95	32	13 ± 1
20	0.96	1.0	1.0	33	17 ± 3
21	3.2	1.0	0.99	33	22 ± 3
22	8.2	1.0	1.0	34	15 ± 1
23	16	0.95	1.1	35	16.5 ± 1
24	8.7	0.93	0.44	14.5	23 ± 3
25	8.3	0.91	2.9	96	24 ± 3
26	8.0	0.87	7.7	250	[c]
27	7.2	0.78	16	542	[c]

[a] Expressed as the total concentration of all species in solution. [b] Each data point represents the average of four to eight determinations; the error limits designate the standard deviation of the determinations. [c] Not measurable.

lent metal ion present in the reaction solutions. This lack of metal ion specificity is emphasized in Figure 6, where the experimental rate constants, k_1*, for all three metal ions are plotted against $1/K_1$(M). All of the data fit quite well on a single straight line.

Creatine Kinase and ATP. Solutions of enzyme and ATP gave single relaxation effects, virtually independent of reactant concentrations over a 3-fold variation in enzyme and a 200-fold variation in ATP concentrations. The data are presented in Table IV. The phenomenon can only be ascribed to an isomerization of the enzyme–substrate complex; no similar effect is observed in the absence of either enzyme or ATP and a bimolecular reaction would certainly exhibit concentration dependence over this wide a concentration range. ATP binds relatively poorly to the enzyme (Kuby *et al.*, 1962) and therein lies the probable cause for the apparent concentration independence of the relaxation time. If a mechanism analogous to eq 1 is assumed, the relationship between τ, rate constants and concentration variables is given by eq 2. The dissociation constant, K_a, is certainly larger for ATP than ADP, so that curvature in plots of $1/\tau$ *vs.* reactant concentrations will become apparent only when measurements are made over a wider concentration range. The values of $1/\tau$ for the lowest reactant concentrations (Table IV) are slightly less than those for higher concentrations, but the imprecision of the data precludes analysis of the type carried out for enzyme–ADP systems.

Nevertheless, some rate limits can be set. Although the relaxation time measured for this system is comparable with that for the enzyme–ADP system, attempts at fitting the data using the previously determined values of k_2 and k_{-2} for ADP binding suggest that the corresponding rate constants for isomerization induced by ATP are somewhat greater. Also, the fact that a relaxation process corresponding to the initial substrate binding step was not seen enables one to set lower limits of $k_1 > 10^7\ M^{-1}\ sec^{-1}$, $k_{-1} > 10^4\ sec^{-1}$ for these constants.

Creatine Kinase, ATP, and Metal Ions (Mg^{2+}, Ca^{2+},

TABLE VI: Rate Constants for Binding of Adenosine Phosphates to Creatine Kinase at 11°.

Substrate	$10^{-7}k_1$ (M^{-1} sec^{-1})	$10^{-4}k_{-1}$ (sec^{-1})	$10^{-4}k_2$ (sec^{-1})	$10^{-4}k_{-2}$ (sec^{-1})
ADP	2.3[a]	1.8[a]	1.67	0.24
MgADP	0.53	0.51	1.67	0.24
CaADP	0.17	0.12	1.67	0.24
MnADP	0.74	0.41	1.67	0.24
ATP	$>10^7$	$>10^4$	$(k_2 + k_{-2}) \geq 2$	
MgATP	$>10^7$	$>10^4$	$(k_2 + k_{-2}) \simeq 2$	
CaATP	$>10^7$	$>10^4$	$(k_2 + k_{-2}) \simeq 2$	
MnATP	$>10^7$	$>10^4$	$(k_2 + k_{-2}) \simeq 2$	

[a] Average of constants from Table III.

or Mn^{2+}). A single relaxation time, analogous to the enzyme–ADP–metal systems and corresponding to an enzyme conformational change, was observed. No concentration dependence was seen for this effect over 4-fold variations in enzyme and 50-fold variations in ATP concentrations nor were significant differences in relaxation rates measured for the three different cations. The experimental results are summarized in Table V. In all experiments the concentration of metal ion was maintained at sufficiently high levels so that nearly all ATP (>90%) was metal ion bound. Therefore, the relaxation effects studied reflect predominantly interactions of enzyme with metal–ATP complexes. Unlike the enzyme–ADP–metal systems, no second relaxation process appeared at low reactant concentrations, indicating that $k_1' > 10^7\ M^{-1}\ sec^{-1}$ and $k_{-1}' > 10^4\ sec^{-1}$ for the enzyme–metal nucleotide association–dissociation process. The value of the reciprocal relaxation time is nearly identical with that found in the enzyme–MADP systems, which suggests that the sum of the rate constants for isomerization, $k_2' + k_{-2}'$, is also the same. However, the lack of concentration dependence of the relaxation time associated with the ATP system indicates that K_a, and therefore the individual rate constants themselves, may not be identical. Unfortunately resolution of the relaxation effect could not be made at sufficiently low reactant concentrations to clarify this issue by determination of K_2.

A summary of all of the rate constants associated with the binding of nucleotide substrates to creatine kinase is given in Table VI.

Creatine Kinase and All Substrates. Reaction solutions containing nearly saturating concentrations of metal–nucleotide and creatine substrates (at equilibrium (MADP) ≃ 2.3 mM, (MATP) ≃ 1.7 mM, (creatine) ≃ 36 mM, (phosphocreatine) ≃ 0.1 mM) gave, in addition to the relaxation times associated with nucleotide binding, two slow relaxation times, one in the millisecond range (τ_3), the other at very long times (τ_4 = seconds).

Stopped-flow experiments were carried out to investigate more carefully the slowest relaxation effect. Relaxation times measured varied with the metal ion activator: for (Mg) = 5.0 mM, τ_4 = 1.6 sec; for (Ca) = 5.0 mM, τ_4 = 1.2 sec; for (Mn) = 1.0 mM, τ_4 = 0.93 sec. Calculations of relaxation times expected for the rate-determining step were made from an abbreviated mechanism which assumed the concentrations of enzyme species containing only one substrate were negligible and considered the formation of the dead-end complex, E–MADP–creatine. For the calculations, maximal reaction velocities for Mg^{2+} were taken from Morrison and James (1965) and corrected approximately for pH (Nihei *et al.*, 1961) and temperature differences. Similarly maximal velocities for the reverse reaction for Ca^{2+} and Mn^{2+} as activator ions were obtained from Morrison and Uhr (1966) and velocities for the forward direction were estimated assuming parallel behavior with Mg^{2+} systems. Such calculations are admittedly crude, but they serve to define the time region for the slowest relaxation process and thus aid the assignment of relaxation effects. The calculations indicate that relaxation times corresponding to the rate-determining step are expected to be in the range of 0.5–5.0 sec with $\tau_{Mg} > \tau_{Ca} > \tau_{Mn}$ for the experimental conditions employed. In addition, the initial velocity for the forward reaction at high enzyme and nearly saturating substrate concentrations with Mg^{2+} as activator ion measured with the stopped-flow apparatus (3×10^{-2} μmole/(μg min), pH 7.6, 29°, 0.1 M KCl) agreed favorably with that determined from steady-state experiments (0.11 μmole/(μg min), pH 8.0, 30°). The lower initial velocity measured by the stopped-flow method can be ascribed largely to pH differences (Nihei *et al.*, 1961) and the fact that substrate saturation of enzyme did not occur in these experiments. Protons were evolved during the equilibrium shift upon temperature perturbation, consistent with $\Delta H°$ values reported for the over-all equilibrium constants (Kuby and Noltmann, 1962). Thus, the combined results indicate strongly that the very slow relaxation process corresponds to the rate-determining step.

The faster relaxation times measured under equilibrium conditions were found to be identical for the three activator ions: for (Mg) = 5.0 mM, τ_3 = 11.5 ± 0.5 msec; for (Ca) = 5.0 mM, τ_3 = 9.2 ± 1.1 msec; for (Mn) = 1.0 mM, τ_3 = 13.5 ± 3.4 msec. These relaxation times are somewhat slower than those measured in the same time range for the analogous blank systems (enzyme absent) for the same experimental conditions (Table I); moreover the relaxation times measured for the metal–adenosine phosphate interactions differ for the various metal ions. Such considerations support the suggestion that the relaxation effect in the presence of enzyme is due to an isomerization process in the ternary complex. In view of the tenuous nature of this assignment and because strong coupling to the metal–nucleotide relaxations, which are at present not understood, might be expected, study of this effect was not pursued.

Discussion

A large amount of information concerning the ligand substitution processes of labile metal ions has been gathered and some general features noted (Eigen and Wilkins, 1964). Rate constants for substitution on a

given metal ion are relatively insensitive to the nature of incoming ligand, but association rates vary widely for different metal ions, suggesting an SN1-type mechanism with dissociation of H_2O from the first-coordination sphere of the metal ion being rate determining (*cf.* Eigen and Tamm, 1962). For the metal ions considered in this study, ligand substitution on Mg^{2+} is at least three orders of magnitude slower than Ca^{2+}, rate constants for substitution on Mn^{2+} being intermediate. This mechanism appears to be operative in biochemical systems as well; binding of pyruvate to Mn–pyruvate carboxylase (Mildvan and Scrutton, 1967) is consistent with the Eigen and Tamm (1962) formulation.

In marked contrast to the behavior expected for metal–ligand substitution reactions, rate constants for metal–ADP associations to creatine kinase are identical within experimental error for the three different activator ions Thus the association process does not appear to involve a normal metal–ligand substitution reaction. A possible explanation is that the enzyme does not bind appreciably to the nucleotide-complexed metal ion. However, the rate constants for metal–ligand substitution are more rapid than for enzyme–substrate association in the cases of Ca^{2+} and Mn^{2+}, and the alternative explanation that metal ions do bridge between enzyme and nucleotide substrate, with some process other than metal–ligand substitution being rate determining, cannot be excluded. This latter possibility seems unlikely on the basis of the results from nuclear magnetic resonance (O'Sullivan and Cohn, 1966a) and electron spin resonance studies (Cohn and Leigh, 1962) from which it was concluded that ligand substitution on Mn^{2+} does not accompany binding of the metal–adenosine phosphate complex to creatine kinase. Comparison with the magnetic resonance results for pyruvate kinase, for which an enzyme–metal–substrate bridge has been demonstrated (Mildvan *et al.*, 1967), considerably augments this interpretation. Ancillary kinetic evidence from steady-state rate investigations has been presented (Morrison and Uhr, 1966; O'Sullivan and Cohn, 1966a) supporting a ternary complex structure with metal binding to nucleotide only.

The dissociation and bimolecular association rate constants for adenosine phosphate binding are comparable with those measured with physiological substrates in other enzyme systems (Hammes, 1968 a,b), being somewhat lower than the value expected for a diffusion-controlled reaction (Alberty and Hammes, 1958). The increasing order of rate constants, k_1, for the substrates, $MADP^- < ADP^{3-} \leq MATP^{2-}$, ATP^{4-} suggests that electrostatic forces might be important factors in determining magnitudes of the association rates. Substrate binding at the enzyme active site might ostensibly then be in the region of loci of positive charges, a reasonable suggestion since the nucleotide substrates carry a large net negative charge. The electrostatic effect is not reflected in K_a values, however, for which the substrate order is estimated as $MATP^{3-} < ADP^{2-} \leq MADP^- < ATP^{4-}$. Results are certainly too fragmentary at present to draw any firm conclusions.

A question which has often been raised in connection with nucleotide binding to creatine kinase is whether or not the nonproductive enzyme–adenosine phosphate complex, once formed, can bind activator metal ion to form the reactive enzyme complex (*cf.* Cleland, 1967), *i.e.*, whether or not the reaction

$$E\text{-}ADP + M \rightleftharpoons E\text{-}MADP$$

occurs. Our analysis indicates that, under equivalent conditions, formation of E–MADP by prior association of free ADP, then metal addition, is more rapid than formation by association of the MADP complex. This result has no particular biological significance; however it is consistent with the formulation of metal binding to nucleotide only.

The wide variety of experiments suggesting the occurrence of enzyme conformational changes upon substrate binding and during the creatine kinase reaction has been alluded to and representative references cited earlier in this paper. The detection of enzyme isomerizations coincident with nucleotide substrate binding by the kinetic methods employed in this study would appear to offer confirmation of these interpretations. Correlation of the isomerizations described kinetically and those indicated by other studies remains to be demonstrated and experiments designed to pursue this question are in progress. Isomerizations accompanying substrate binding have been reported for several enzyme systems (Hammes, 1968 a,b). The relaxation times, τ_2, measured for adenosine phosphate binding in this instance are quite similar in magnitude to those previously determined ($\tau = 10^{-3}$–10^{-4} sec). The rate of conformational changes induced in creatine kinase by ADP substrates is independent of the presence of activator ion in addition to being the same for the three metal ions. Again, this nonparticipation of activator cation in the isomerization process is consistent with the enzyme–metal–nucleotide structure proposed.

From a study of the effect of nucleotides and their magnesium complexes upon the alkylation rates of creatine kinase, O'Sullivan and Cohn (1966b) concluded that the conformational change induced by binding of free nucleotides was different from that induced by the metal–nucleotide complexes. Our analysis of the enzyme isomerizations caused by ADP and metal–ADP binding reveals that the rates of isomerization are essentially identical for the various adenosine diphosphate substrates. The circumstance that different isomerization processes have quite similar rate constants seems plausible, especially if the measured rate constants are a function principally of the cooperative nature of the isomerization (Hammes, 1968 a,b). Of course, the explanation that two isomerizations exist, one affecting predominantly sulfhydryl group reactivities, the other, acid dissociation constants of side chain residues, is equally tenable.

Inhibition of the enzymatic reaction by high concentrations of activator ion has been reported (Morrison and Uhr, 1966; Morrison and O'Sullivan, 1965). Inhibition by Mg^{2+} with respect to MgADP is noncompetitive, while that by Ca^{2+} and Mn^{2+} is uncompetitive with respect to the corresponding metal–nucleotide complexes. For all three ions, inhibition is noncompetitive with

respect to phosphocreatine. Thus all three metal ions are capable of interacting with enzyme–MADP complexes, while only Mg^{2+} can interact with the free form of the enzyme to produce inhibition. This behavior observed for steady-state conditions may relate to the disappearance of enzyme isomerizations (τ_2) accompanying MATP binding at high Ca^{2+} and Mn^{2+} concentrations noted previously.

Binding of creatine base substrates to the enzyme apparently produces no reciprocal conformational change. However, these observations are quite uncertain, since for phosphocreatine, a relaxation effect might easily have been missed because CrP buffers the reaction solution in the pH region of the studies, and for creatine, only about 25–50% enzyme saturation was reached at the highest substrate concentrations employed (K_m = 10–50 mM (Morrison and James, 1968; O'Sullivan *et al.*, 1966; Jacobs and Cunningham, 1968)).

The assignment of an isomerization of the ternary complex for τ_3 is possible, but is certainly not proven by the available data. The relative slowness of the relaxation process might be taken to indicate a highly cooperative process (*cf.* Hammes, 1968 a,b), which in turn implies extensive dislocations in the protein. Changes in optical rotation and immunochemical specificity, parameters which reflect the gross protein structure, are observed for creatine kinase only in the presence of both adenosine phosphate and creatine substrates (Samuels *et al.*, 1961). O'Sullivan and Cohn (1968) have noted that the entropy for creatine binding to the enzyme–MnADP complex, −40 eu, is consistent with a contribution from an enzyme conformational change. However, differences in other physicochemical parameters, *e.g.*, sedimentation coefficients, H–D exchange rates, rates of tryptic digestion and intrinsic viscosities, were insignificant when measurements on the "working" enzyme were compared with those for the enzyme when all substrates were not present, suggesting to the investigators that protein conformational changes during reaction are not extensive (Lui and Cunningham, 1966).

Postulation of a detailed chemical mechanism for the action of creatine kinase on the basis of these data is not warranted. However, these results clearly indicate the prevalence of conformational changes (or isomerizations) in the interaction of creatine kinase with its substrates, and show that a discrimination with respect to the metal ion is shown mainly in the rate-determining step.

Appendix

The mechanism of eq 3 is characterized by five relaxation times. When the vertical steps (the ligand–metal reactions) equilibrate rapidly, each vertical step is characterized by a relaxation time of the form $1/\tau = k_a((\overline{M}) + (\overline{L})) + k_d$, where k_a is the association constant, k_d is the dissociation constant, and $(\overline{L})$ is the equilibrium ligand concentration. The approach to equilibrium of the horizontal steps is characterized by the two remaining relaxation times (*cf.* Hammes and Schimmel, 1966). This system can be described by the rate equations

$$-\frac{d(E)}{dt} = k_1(E)(ADP) + k_1'(E)(MADP) - k_{-1}(X_1) - k_{-1}'(X_1M) \quad (A1)$$

$$-\frac{d[(X_2) + (X_2M)]}{dt} = k_{-2}(X_2) + k_{-2}'(X_2M) - k_2(X_1) - k_2'(X_1M) \quad (A2)$$

where $X_1 = (EADP)_1$ and $X_2 = (EADP)_2$. In the neighborhood of equilibrium these equations can be written as (*cf.* Amdur and Hammes, 1966)

$$-\frac{d\delta(E)}{dt} = k_1[(\overline{E})\delta(ADP) + (\overline{ADP})\delta(E)] + k_1'[(\overline{MADP})\delta(E) + (\overline{E})\delta(MADP)] - k_{-1}\delta(X_1) - k_{-1}'\delta(X_1M) \quad (A3)$$

$$-\frac{d[\delta(X_2) + \delta(X_2M)]}{dt} = k_{-2}\delta(X_2) + k_{-2}'\delta(X_2M) - k_2\delta(X_1) - k_2'\delta(X_1M) \quad (A4)$$

where δ represents the deviation from equilibrium and the bars denote equilibrium concentrations. Conservation of mass requires that

$$\delta(E) + \delta(X_1) + \delta(X_2) + \delta(X_1M) + \delta(X_2M) = 0 \quad (A5)$$

$$\delta(M) + \delta(MADP) + \delta(X_1M) + \delta(X_2M) = 0 \quad (A6)$$

$$\delta(E) = \delta(ADP) + \delta(MADP) \quad (A7)$$

In addition the equilibria represented by the vertical steps are assumed to always be at equilibrium. The equilibrium constants are

$$K_1 = (MADP)/(M)(ADP) \quad (A8)$$

$$K_2 = (X_1M)/(M)(X_1) \quad (A9)$$

$$K_3 = (X_2M)/(M)(X_2) \quad (A10)$$

and differentiation of these equations gives

$$K_1(ADP)\delta(M) + K_1(M)\delta(ADP) = \delta(MADP) \quad (A11)$$

$$K_2(X_1)\delta(M) + K_2(M)\delta(X_1) = \delta(X_1M) \quad (A12)$$

$$K_3(X_2)\delta(M) + K_3(M)\delta(X_2) = \delta(X_2M) \quad (A13)$$

Substitution of eq A5 and A7 in A3 and A4 gives

$$-\frac{d\delta(E)}{dt} = \{k_1[(\overline{ADP}) + (\overline{E})/(1 + \delta(MADP)/\delta(ADP))] + k_1'[(\overline{MADP}) + (E)/(1 + \delta(ADP)/\delta(MADP))] + k_{-1}/(1 + \delta(X_1M)/\delta(X_1)) + k_{-1}'/(1 + \delta(X_1)/\delta(X_1M))\}\cdot\delta(E) + \{k_{-1}/(1 + \delta(X_1M)/\delta(X_1)) + k_{-1}'/(1 + \delta(X_1)/\delta(X_1M))\}(\delta(X_2) + \delta(X_2M)) \quad (A14)$$

$$-\frac{d(\delta(X_2) + \delta(X_2M))}{dt} = [k_2/(1 + \delta(X_1M)/\delta(X_1)) + k_2'/(1 + \delta(X_1)/\delta(X_1M))]\delta(E) + [k_{-2}/(1 + \delta(X_2M)/\delta(X_2)) + k_{-2}'/(1 + \delta(X_2)/\delta(X_2M)) + k_2/(1 + \delta(X_1M)/\delta(X_1)) + k_2'/(1 + \delta(X_1)/\delta(X_1M))](\delta(X_2) + \delta(X_2M)) \quad (A15)$$

For the experimental conditions of relatively high metal ion concentrations, the approximation $\delta(M) = 0$ is valid and eq A11–A13 reduce to

$$K_1(M) = \delta(MADP)/\delta(ADP)$$
$$K_2(M) = \delta(X_1M)/\delta(X_1)$$
$$K_3(M) = \delta(X_2M)/\delta(X_2)$$

Substitution of eq A11–A13 into A14 and A15 and use of eq A8–A10 gives

$$-\frac{d\delta(E)}{dt} = a_{11}\delta(E) + a_{12}(\delta(X_2) + \delta(X_2M))$$

$$-\frac{d(\delta(X_2) + \delta(X_2M))}{dt} = a_{21}\,\delta(E) + a_{22}(\delta(X_2) + \delta(X_2M))$$

where the a_{ij} are those defined in the text.

If $k_2 = k_2'$ and $k_{-2} = k_{-2}'$, as inferred from the data, a number of relationships can be derived from the principle of detailed balance. Since $k_2'/k_{-2}' = k_2/k_{-2}$, $K_2 = K_3$. Similarily since $K_B = k_{-1}/k_1\,(1 + (k_2/k_{-2}))$ and $K_{MB} = k_{-1}'/k_1'\,(1 + (k_2'/k_{-2}'))$, $K_B/K_{MB} = (k_{-1}/k_1)/(k_{-1}'/k_1') = K_2/K_1$. These relationships can be used to show that $k_{-1}^* = k_1^* K_{MB}(1 + K_b)$.

References

Alberty, R. A., and Hammes, G. G. (1958), *J. Phys. Chem. 62*, 154.

Amdur, I., and Hammes, G. G. (1966), Chemical Kinetics, New York, N. Y., McGraw-Hill, p 138.

Burton, K. (1959), *Biochem. J. 71*, 388.

Castellan, G. W. (1963), *Ber. Bunsenges. Physik. Chem. 67*, 898.

Cleland, W. W. (1967), *Ann. Rev. Biochem. 36*, 96.

Cohn, M., and Leigh, J. S. (1962), *Nature 193*, 1037.

Diebler, H., Eigen, M., and Hammes, G. G. (1960), *Z. Naturforsch. 15b*, 554.

Eigen, M., and Hammes, G. G. (1961), *J. Am. Chem. Soc. 83*, 2786.

Eigen, M., and Tamm, K. (1962), *Z. Elektroc. n. 66*, 107.

Eigen, M., and Wilkins, R. G. (1964), Mechanisms of Inorganic Reactions, Advances in Chemistry Series No. 49, Washington, D. C., American Chemical Society, p 55.

French, T. C., and Hammes, G. G. (1969), *Methods Enzymol.* (in press).

Hammes, G. G. (1968a), *Accounts Chem. Res. 1*, 321.

Hammes, G. G. (1968b), *Advan. Protein Chem. 23*, 1.

Hammes, G. G., and Schimmel, P. R. (1966), *J. Phys. Chem. 70*, 2319.

Jacobs, G., and Cunningham, L. W. (1968), *Biochemistry 7*, 143.

James, E., and Morrison, J. F. (1966), *J. Biol. Chem. 241*, 4758.

Kahn, M. M. T., and Martell, A. E. (1966), *J. Am. Chem. Soc. 88*, 668.

Kahn, M. M. T., and Martell, A. E. (1967), *J. Am. Chem. Soc. 89*, 5585.

Kuby, S. A., Mahowald, T. A., and Noltmann, E. A. (1962), *Biochemistry 1*, 748.

Kuby, S. A., Noda, L., and Lardy, H. A. (1954), *J. Biol. Chem. 210*, 191.

Kuby, S. A., and Noltmann, E. A. (1962), *Enzymes 6*, 516.

Lui, N. S. T., and Cunningham, L. W. (1966), *Biochemistry 5*, 144.

Mahowald, T. A., Noltmann, E. A., and Kuby, S. A. (1962), *J. Biol. Chem. 237*, 1535.

Mildvan, A. S., Leigh, J. S., and Cohn, M. (1967), *Biochemistry 6*, 1805.

Mildvan, A. S., and Scrutton, M. C. (1967), *Biochemistry 6*, 2978.

Morrison, J. F., and Cleland, W. W. (1966), *J. Biol. Chem. 241*, 673.

Morrison, J. F., and James, E. (1965), *Biochem. J. 97*, 37.

Morrison, J. F., and O'Sullivan, W. J. (1965), *Biochem. J. 94*, 221.

Morrison, J. F., and Uhr, M. L. (1966), *Biochim. Biophys. Acta 122*, 57.

Morrison, J. F., and White, A. (1967), *European J. Biochem. 3*, 145.

Nihei, T., Noda, L., and Morales, M. F. (1961), *J. Biol. Chem. 236*, 3203.

O'Sullivan, W. J., and Cohn, M. (1966a), *J. Biol. Chem. 241*, 3104.

O'Sullivan, W. J., and Cohn, M. (1966b), *J. Biol. Chem. 241*, 3116.

O'Sullivan, W. J., and Cohn, M. (1968), *J. Biol. Chem. 243*, 2737.

O'Sullivan, W. J., Diefenbach, H., and Cohn, M. (1966), *Biochemistry 5*, 2666.

O'Sullivan, W. J., and Perrin, D. D. (1964), *Biochemistry 3*, 18.

Samuels, A. J., Nihei, T., and Noda, L. (1961), *Proc. Natl. Acad. Sci. U. S. 47*, 1992.

Watts, D. C. (1963), *Biochem. J. 89*, 220.