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Kinetics of Inactivation of Creatine Kinase during Modification of Its Thiol Groups†

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ABSTRACT: Kinetics of inactivation and modification of the reactive thiol groups of creatine kinase by 5,5′-dithiobis(2-nitrobenzoic acid) or iodoacetamide have been compared, the former by following the substrate reaction in presence of the inactivator [Wang, Z.-X., & Tsou, C.-L. (1987) *J. Theor. Biol. 127*, 253]. The microscopic constants for the reaction of the inactivators with the free enzyme and with the enzyme–substrate complexes were determined. From the results obtained it appears that with respect to ATP both inactivators are noncompetitive whereas for creatine iodoacetamide is competitive but DTNB is not. The formation of the ternary complex protects against the inactivation by both DTNB and iodoacetamide. The inactivation kinetics is monophasic with both inactivators, but under similar conditions, the modification reactions in the presence of the transition-state analogue of creatine–ADP–Mg^{2+}–nitrate show biphasic kinetics as also reported by Price and Hunter [Price, N. C., & Hunter, M. G. (1976) *Biochim. Biophys. Acta 445*, 364]. If the reactive ternary complex and the enzyme complexed with the transition-state analogue react in the same way with these reagents, the modification of one fast-reacting thiol group for each enzyme molecule leads to complete inactivation, indicating that the enzyme has to be in the dimeric state to be active.

Some years ago, a systematic study on the kinetics of irreversible modification of enzyme activity was presented [Tsou, 1965a,b; see Tian and Tsou (1982)]. From the equations derived for the substrate reaction in the presence of the modifier, the rate constant for the irreversible inhibition of enzyme activity can be obtained in one single experiment. Recently, this method has been employed for the determination of the rate constants of irreversible modification of a number of enzymes in different laboratories (Tian & Tsou, 1982; Bieth, 1984; Harper & Powers, 1984; Harper et al., 1985; Mason et al., 1985; Liu & Tsou, 1986). In the above studies, the kinetics of irreversible inhibition of enzymes reacting with a single substrate was considered, and the kinetic treatment has since been extended to enzyme reactions involving two substrates (Wang & Tsou, 1987).

Creatine kinase (EC 2.7.3.2) is a dimeric enzyme composed of identical subunits with eight Cys residues (Putney et al., 1984). Of the eight thiol groups of the native enzyme, only two are capable of reacting with a number of modification reagents, and the modified cysteine residues are situated at the same position in the primary sequence of each subunit (Zhou & Tsou, 1987). They are believed to be essential for the activity of the enzyme on the basis of extensive modification studies with a large number of reagents including *N*-ethylmaleimide (Ennor & Rosenberg, 1954), iodoacetic acid (Watts & Rabin, 1962), iodoacetamide and derivatives (Price, 1979), 2,4-dinitrofluorobenzene (O'Sullivan & Chen, 1966), (dimethylamino)naphthalenesulfonyl chloride (Brown & Cunningham, 1970), DTNB[1] (Price & Hunter, 1976), and iodomethane (Reddy & Watts, 1979). It has also been reported that substrates protect these thiol groups from modification (Watts & Rabin, 1962; Price & Hunter, 1976), and a mechanism of action of this enzyme with an SH group in each subunit taking an active part was proposed by Watts and Rabin (1962). It was also reported that the reaction between these two thiol groups with some reagents shows biphasic kinetics in the presence of the transition-state analogue creatine–ADP–Mg^{2+}–NO_3^-. On the basis of some intriguing experiments in which the two reactive thiol groups behave differently, Degani and Degani (1979) suggested that the two subunits of this enzyme are asymmetrically arranged and the

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[1] Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Cr, creatine (used only in equations).

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two active site thiol groups play different roles. It would therefore be of interest to compare the kinetics of inactivation with the kinetics of modification of the active site thiol groups. Results reported in the present paper show that although the modification reaction with DTNB is biphasic in the presence of the transition-state analogue as reported previously by Price and Hunter (1976), the kinetics of inactivation with both DTNB and iodoacetamide as followed by the substrate reaction in the presence of the modifiers is monophasic. It is suggested that the modification of one of the two reactive thiols leads to complete inactivation. The relevant microscopic rate constants for the reactions of iodoacetamide and DTNB with the various enzyme–substrate complexes were also determined.

Materials and Methods

The preparation and assay of rabbit muscle creatine kinase were as described before (Yao et al., 1982) except that bovine serum albumin was omitted from the assay mixture. The reaction of ATP with creatine results in the release of a proton which was followed by measurement of the absorbance change at 597 nm of the pH indicator thymol blue. The reaction mixture, 2 mL, contained 24 mM creatine, 4 mM ATP, 5 mM Mg^{2+}, and 0.01% thymol blue, in 5 mM glycine–NaOH buffer, pH 9.0. The reaction mixture was carefully adjusted to pH 9.0 before use, and a calibration curve was constructed to correlate the amount of proton generated and the absorbance change at 597 nm under the above conditions. Creatine kinase has a broad optimum of pH 8.0–9.0 (Watts, 1973), and a change of 0.5 pH unit in this range has no appreciable effect on its activity. Enzyme concentration was determined by absorbance at 280 nm with $A^{1\%}_{1cm} = 8.8$ (Noda et al., 1954). DTNB was from Sigma, ATP from Boeringer Mannheim GmbH, ADP from Serva, and creatine from E. Merck. Iodoacetamide was a local product and recrystallized twice before use. Modification of SH groups by DTNB was carried out in 50 mM glycine–NaOH buffer, pH 9.0, at 25 °C and followed by absorbance changes at 412 nm due to the generation of 2-mercapto-5-nitrobenzoic acid, and a molar absorption coefficient of 13 600 (Ellman, 1959) was used to calculate its rate of generation. The activity determinations and the absorption measurements were carried out with a Cary 219 spectrophotometer.

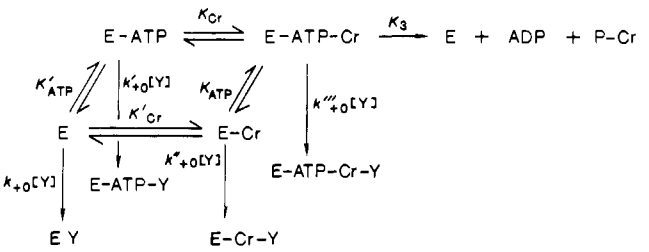
The kinetics of the inactivation reaction was followed by the substrate reaction in the presence of the modifier as described before (Tsou, 1965b; Tian & Tsou, 1982; Wang & Tsou, 1987). For comparison, in some of the experiments, the inactivation rate constants were also determined in the conventional way essentially according to Watts and Rabin (1962) except that, instead of stopping the reaction of the enzyme with the modifier by an excess of a thiol, 20 μL of the reaction mixture containing 3.5 μM enzyme and 0.1 mM iodoacetamide in 50 mM Gly–NaOH buffer at pH 9.0 was diluted directly into 2 mL of the assay mixture. With a dilution of 100-fold, further progress of the bimolecular reaction between the enzyme and the modifier became negligible. All measurements were carried out at 25 °C.

Kinetic Analysis

Detailed kinetic analysis of the substrate reaction during irreversible inhibition of an enzyme involving two substrates has been presented (Wang & Tsou, 1987) and reviewed recently (Tsou, 1988). Only the relevant points will be summarized below. The concentration of product formed at time t, [P], is given by

$$[P] = [P]_\infty(1 - e^{-A[Y]t}) \quad (1)$$

Scheme I



in which $[P]_\infty$ is the product concentration at time infinity, A is the apparent rate constant of inactivation, and [Y] is the concentration of the modifier. For enzyme reactions involving two substrates with random substrate binding sequence in rapid equilibrium as shown for creatine kinase (Morrison & James, 1965; Maggio & Kenyon, 1977) in Scheme I, $[P]_\infty$ and $1/A$ are given respectively by

$$[P]_\infty = \frac{v}{A[Y]} = k_3[E]_0[ATP][Cr]/(k_{+0}K'_{ATP}K_{Cr} + k'_{+0}K_{Cr}[ATP] + k''_{+0}K_{ATP}[Cr] + k'''_{+0}[ATP][Cr])[Y] \quad (2)$$

$$1/A = (K'_{ATP}K_{Cr} + K_{Cr}[ATP] + K_{ATP}[Cr] + [ATP][Cr])/(k_{+0}K'_{ATP}K_{Cr} + k'_{+0}K_{Cr}[ATP] + k''_{+0}K_{ATP}[Cr] + k'''_{+0}[ATP][Cr]) \quad (3)$$

From eq 1 and 2 it can be seen that at time infinity [P] approaches constant values which decrease with increasing values of [Y], and plots of ln ($[P]_\infty$ − [P]) against t give straight lines with slopes of $-A[Y]$ (eq 4).

$$\ln([P]_\infty - [P]) = \ln[P]_\infty - A[Y]t \quad (4)$$

Differentiation of Noncomplexing and Complexing Inhibitions. It has been considered necessary to make a distinction between noncomplexing and complexing types of irreversible inhibitions. For the complexing type of inhibitions, the rapid formation of a noncovalent enzyme–inhibitor complex precedes the slow irreversible modification step. As has been shown before (Wang & Tsou, 1987), the expressions for the apparent rate constants are different for complexing and noncomplexing inhibitions in that the expression for the apparent rate constant A contains the term [Y] for complexing inhibitions whereas it is independent of [Y] for noncomplexing inhibitions. This provides the basis for the experimental differentiation of these two types of inhibitions. As A is independent of [Y] for noncomplexing inhibitions, a plot of $1/A$ against [Y] gives a straight line parallel to the abscissa whereas $1/A$ increases with increasing [Y] and the straight line cuts at the ordinate for complexing inhibitions.

Determination of the Microscopic Rate Constants. Equation 2 can be written as

$$\frac{1}{[P]_\infty} = \left(\frac{k_{+0}[Y]K'_{ATP}K_{Cr}}{k_3[E]_0[ATP]} + \frac{k'_{+0}[Y]K_{Cr}}{k_3[E]_0}\right)\frac{1}{[Cr]} + \frac{k''_{+0}[Y]K_{ATP}}{k_3[E]_0[ATP]} + \frac{k'''_{+0}[Y]}{k_3[E]_0} \quad (5)$$

and it can be seen from the above that, while keeping [ATP] constant, a plot of $1/[P]_\infty$ against 1/[Cr], should give a straight line (Figures 3 and 5) with a slope s and an ordinate intercept i of

$$s = \frac{k_{+0}[Y]K'_{ATP}K_{Cr}}{k_3[E]_0[ATP]} + \frac{k'_{+0}[Y]K_{Cr}}{k_3[E]_0} \quad (6)$$

$$i = \frac{k''_{+0}[Y]K_{ATP}}{k_3[E]_0[ATP]} + \frac{k'''_{+0}[Y]}{k_3[E]_0} \quad (7)$$

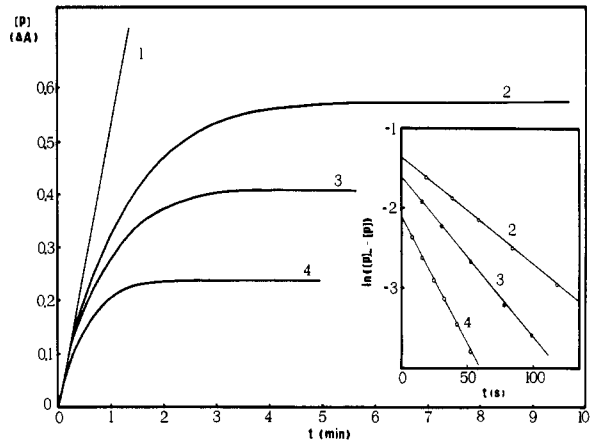


FIGURE 1: Substrate reaction of creatine kinase in the presence of iodoacetamide. The reaction mixture contained 5 mM Gly–NaOH buffer, pH 9.0, 0.01% thymol blue, 24 mM creatine, 4 mM ATP–Mg^{2+}, 1 mM Mg^{2+}, and 29 nM rabbit muscle creatine kinase. Concentrations of iodoacetamide were 0, 2, 2.9, and 4.8 mM, respectively, for curves 1–4. Proton generation was followed by the absorbance change, 597 nm, of the indicator at 25 °C. The inset shows semilogarithmic plots of lines 2–4 according to eq 4.

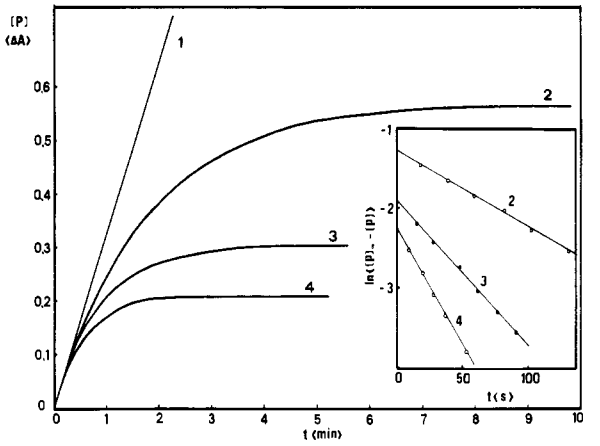


FIGURE 2: Substrate reaction of creatine kinase in the presence of DTNB. The reaction mixture contained 5 mM Gly–NaOH buffer, pH 9.0, 0.01% thymol blue, 24 mM creatine, 4 mM ATP–Mg^{2+}, 1 mM Mg^{2+}, and 30 nM creatine kinase. Concentrations of DTNB were 0, 1.5, 3, and 4.5 μM, respectively, for curves 1–4. Proton generation was followed by the absorbance change, 597 nm, of the indicator at 25 °C. The inset shows semilogarithmic plots of lines 2–4 according to eq 4.

Both the slope and the intercept are linear functions of $1/[ATP]$. Secondary plots of s against $1/[ATP]$ give respectively the values of the intercepts on the ordinate and the slopes as

$$\text{intercept} = k'_{+0}K_{Cr}[Y]/k_3[E]_0 \quad (8)$$

$$\text{slope} = k_{+0}K'_{ATP}K_{Cr}[Y]/k_3[E]_0 \quad (9)$$

and for the i against $1/[ATP]$ plot

$$\text{intercept} = k'''_{+0}[Y]/k_3[E]_0 \quad (10)$$

$$\text{slope} = k''_{+0}K_{ATP}[Y]/k_3[E]_0 \quad (11)$$

From these values it can be seen that since k_3 and the respective dissociation constants can be obtained in experiments without the inhibitor, the microscopic rate constants, k_{+0}, k'_{+0}, etc., can then be calculated.

RESULTS

Inactivation Kinetics of Creatine Kinase with Iodoacetamide. The course of substrate reaction in the presence of different concentrations of iodoacetamide is shown in Figure 1. It can be seen that, as predicted from eq 2, [P] approach constant final values, $[P]_\infty$, which decrease with increasing concentrations of iodoacetamide. Semilogarithmic plots according to eq 4 are given in the inset of Figure 1, and the apparent rate constant can then be calculated from the slopes of the straight lines obtained. Results presented in the Figure 1 inset also show that the inactivation is a monophasic pseudo-first-order reaction. A plot of $1/A$ against iodoacetamide concentration (1.6–4.8 mM) shows that the apparent rate constant A is not affected by iodoacetamide concentration (data no shown), indicating that there is no prior complexing with the enzyme before the irreversible modification step (Tian & Tsou, 1982; Wang & Tsou, 1987).

Inactivation Kinetics of Creatine Kinase with DTNB. The inactivation of the enzyme by DTNB is much faster than that by iodoacetamide. However, both inactivation reactions show similar kinetic characteristics with $[P]_\infty$ approaching constant final values that decrease at higher concentrations of the inactivator (Figure 2). The monophasic nature of the pseudo-first-order inactivation reaction with DTNB is shown in semilogarithmic plots given in the inset of Figure 2. The apparent rate constants can be calculated from the slopes of the straight lines. DTNB, like iodoacetamide, is also a non-complexing irreversible inhibitor as shown by a plot of $1/A$ against DTNB concentration (1.4–12 μM, data not shown).

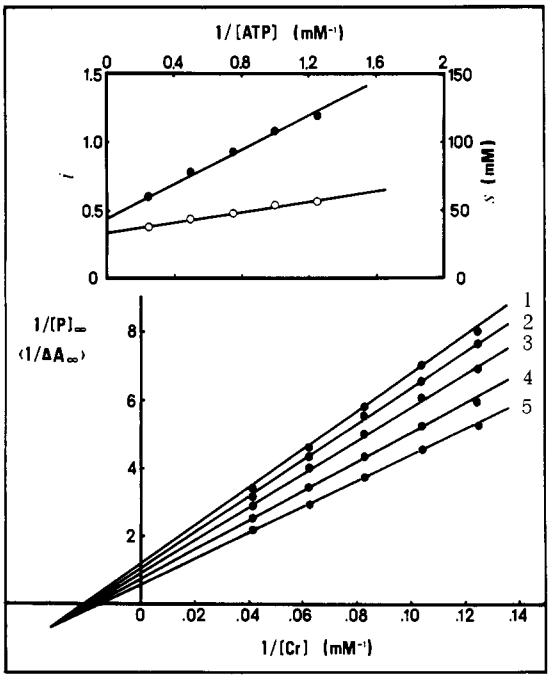


FIGURE 3: Effect of creatine concentration on $[P]_\infty$ during inactivation by iodoacetamide. Experimental conditions as for Figure 1 except concentrations for iodoacetamide and creatine kinase were 2.5 and 48 nM, respectively, and for creatine and ATP were as indicated. Double-reciprocal plots of $1/[P]_\infty$ against $1/[Cr]$ with [ATP] of 0.8, 1.0, 1.33, 2.0, and 4.0 mM for lines 1–5, respectively. The inset shows the secondary plots of the ordinate intercepts (full circles and left-hand scale) and the slopes (open circles and right-hand scale) against $1/[ATP]$.

Effect of Substrate on the Inactivation by Iodoacetamide. Substrate protection against inactivation of creatine kinase by iodoacetamide and DTNB was reported before (Watts & Rabin, 1962; Price & Hunter, 1976) with no quantitative determination on the extent of protection. With the present approach, from the measurements of the substrate reaction in the presence of the modifier at different substrate concentrations, the rate constants k_{+0}, k'_{+0}, k''_{+0}, and k'''_{+0} can all be obtained from suitable plots according to eq 5–11. Figures 3 and 4 show the respective plots of $1/[P]_\infty$ against $1/[Cr]$

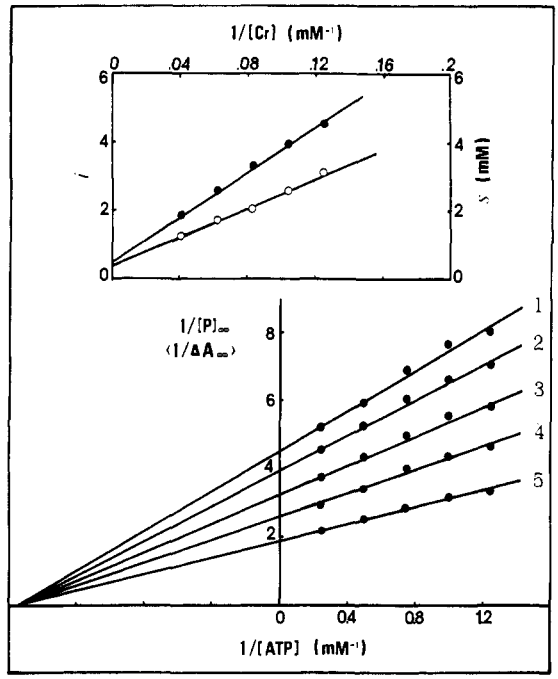


FIGURE 4: Effect of ATP concentration on $[P]_\infty$ during inactivation by iodoacetamide. Experimental conditions as for Figure 3 except creatine and ATP concentrations were as indicated. Double-reciprocal plots of $1/[P]_\infty$ against $1/[ATP]$ with [Cr] of 8, 9.6, 12, 16, and 24 mM for lines 1–5, respectively. The inset shows the secondary plots of the ordinate intercepts (full circles and left-hand scale) and the slopes (open circles and right-hand scale) against $1/[Cr]$.

Table I: Kinetic Parameters of Creatine Kinase Obtained by Initial Velocity Measurements[a]

Michaelis constants	
K'_{Cr} (mM)	6.9
K'_{ATP} (mM)	0.6
K_{Cr} (mM)	15
K_{ATP} (mM)	1.2
max velocity [mol/(min·mg)]	138

[a] The Michaelis constants and maximal velocity for pH 9.0 and 25 °C were obtained from Lineweaver and Burk plots and suitable secondary plots (Morrison & James, 1965) of initial velocities measured in the presence of different concentrations of creatine and ATP. Equilibrium constants, as defined by Wang and Tsou (1987), are K'_{ATP}[E–ATP] = [E][ATP], K'_{Cr}[E–Cr] = [E][Cr], and $K'_{ATP}K_{Cr}$[E–ATP–Cr] = $K'_{Cr}K_{ATP}$[E–ATP–Cr] = [E][ATP][Cr].

and $1/[ATP]$ as well as the secondary plots according to eq 6 and 7. In order to obtain the microscopic rate constants for the inactivation reactions, it is necessary to have the respective K_M and V_{max} values for the uninhibited reaction under otherwise identical conditions (pH 9.0 and 25 °C). These were obtained from Lineweaver and Burk plots and suitable secondary plots (Morrison & James, 1965) of initial velocity measurements in the presence of different concentrations of creatine and ATP but in the absence of added modifiers (data not shown). The values thus obtained (Table I) are in accord with those given by Maggio and Kenyon (1977) for pH 9.0 and 30 °C. From these and the secondary plots given in the insets of Figures 3 and 4, the microscopic rate constant for the reaction of iodoacetamide with the free enzyme and the E–ATP, E–creatine, and E–ATP–creatine complexes can then be obtained (eq 8–11), and the results are listed in Table II. For comparison, the inactivation rate was also determined by the conventional method, giving a second-order rate constant of 12 M^{-1} s^{-1} as compared to a value of 7.6 obtained from the plot in the inset of Figure 3. Both values are listed in Table II. It can be seen that iodoacetamide is competitive with

Table II: Rate Constants of the Reactions of Creatine Kinase with Iodoacetamide and DTNB[a]

form of enzyme reacting		second-order rate constant (M^{-1} s^{-1})	
		iodoacetamide	DTNB ($\times 10^{-4}$)
E	k_{+0}	7.6	1.1
		12.0[b]	4.4[c]
		9.0[d]	
E–ATP	k'_{+0}	14.4	1.7
E–Cr	k''_{+0}	2.2	1.1
E–ATP–Cr	k'''_{+0}	1.5	<0.05

[a] All the rate constants were obtained by following the substrate reaction in the presence of the inactivator as described in the text except where noted. For details, see text. [b] Obtained by the conventional method of taking aliquots of the reaction mixture at time intervals and assaying for enzyme activity. [c] Rate constant for the modification reaction measured directly at 412 nm. [d] Value taken from Price (1979).

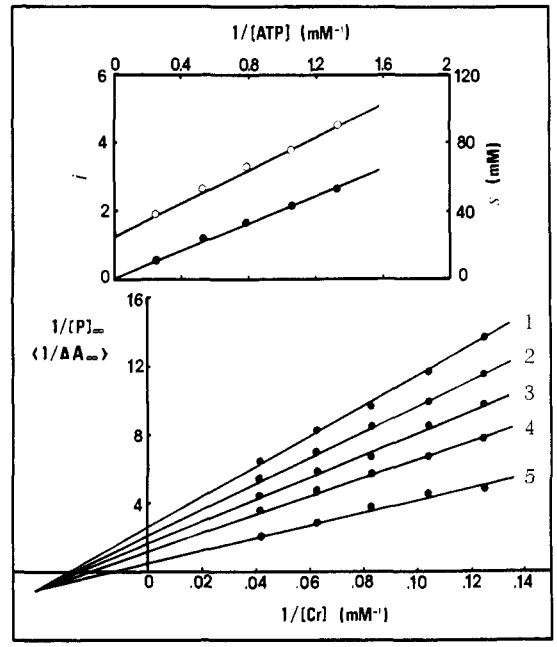


FIGURE 5: Effect of creatine concentration on $[P]_\infty$ during DTNB inactivation. Experimental conditions as for Figure 2 except the enzyme and DTNB concentrations were 48 nM and 3 μM, respectively. The creatine and ATP concentrations were as indicated. Double-reciprocal plots are of $1/[P]_\infty$ against $1/[Cr]$, and the [ATP] were, for lines from top to bottom, 0.75, 0.94, 1.26, 1.88, and 3.77 mM. The inset shows the secondary plots of the ordinate intercepts (full circles and left-hand scale) and the slopes (open circles and right-hand scale) against $1/[ATP]$.

respect to creatine but noncompetitive to ATP. The formation of the ternary complex gives further protection against iodoacetamide inhibition.

Effect of Substrate on the Inactivation Rate with DTNB. The effects of different concentrations of creatine and ATP on the inactivation rate with DTNB have also been studied. Figures 5 and 6 show the plot of $1/[P]_\infty$ against $1/[Cr]$ and $1/[ATP]$, respectively, as well as the secondary plots according to eq 6 and 7. The respective microscopic rate constants similarly obtained as above are several orders of magnitude greater than those for the iodoacetamide reaction as also listed in Table II. The rate constant thus obtained for the reaction of DTNB with the free enzyme, 1.1×10^4 M^{-1} s^{-1}, is of the same order of magnitude to that obtained separately by direct reaction of DTNB with the enzyme, 4.4×10^4 M^{-1} s^{-1}. The difference is probably due to slightly different experimental conditions. It is to be noted that either ATP or creatine alone does not affect the rate constant of the reaction between the enzyme and DTNB which is therefore noncompetitive with

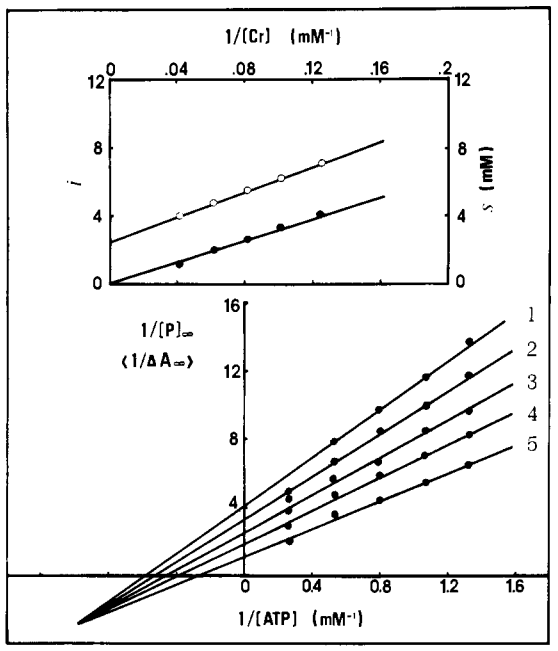


FIGURE 6: Effect of ATP concentration on $[P]_\infty$ during DTNB inactivation. Experimental conditions as for Figure 3 except creatine and ATP concentrations are as indicated. Double-reciprocal plots are of $1/[P]_\infty$ against $1/[ATP]$, and the [Cr] were, for lines from top to bottom, 8, 9.6, 12, 16, and 24 mM. The inset shows the secondary plots of the the ordinate intercepts (full circles and left-hand scale) and the slopes (open circles and right-hand scale) against $1/[Cr]$.

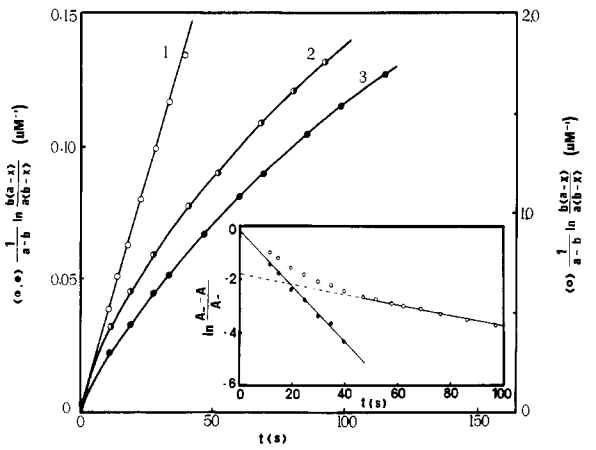


FIGURE 7: Reaction of creatine kinase with DTNB in the presence of the transition-state analogue. The reaction mixture contained (1) 1.2 μM creatine kinase and 3 μM DTNB in 50 mM Gly–NaOH buffer, pH 9.0 (right-hand scale); (2) 3 μM creatine kinase, 25 μM DTNB, 30 mM creatine, 2 mM ADP, 2 mM Mg^{2+}, and 10 mM nitrate (left-hand scale); and (3) as in (2) but without Mg^{2+} (left-hand scale). The reaction was followed at 412 nm and plotted as a second-order reaction. Temperature was 25 °C. The inset shows a semilogarithmic plot of the pseudo-first-order reaction of creatine kinase with excess DTNB in the prescence of the transition-state analogue. The reaction mixture contained 1.3 μM enzyme, 50 μM DTNB, 30 mM creatine, 2 mM ADP, and 10 mM nitrate. Open circles, direct plot; full circles, fast phase of the reaction.

respect to both substrates. However, the formation of the ternary complex affords extensive protection against DTNB inhibition.

Rate of Modification of the Reactive Thiol Groups of Creatine Kinase. The modification of creatine kinase by DTNB and iodoacetic acid has been carefully studied by Price and Hunter (1976). The reaction is monophasic, showing that the reactive thiols of both the subunits react at the same rate. However, in the presence of the transition-state analogue, ADP–Mg^{2+}–creatine–NO_3^-, the reaction becomes biphasic. In order to compare the kinetics of modification with that of inactivation under similar conditions, the direct reaction of DTNB with the enzyme was followed at the same pH and temperature as employed for the study of the inactivation kinetics, and the results are shown in Figure 7. In agreement with Price and Hunter (1976), the modification of the free enzyme with DTNB is a monophasic reaction, but in the presence of the transition-state analogue, the second-order plot of the modification reaction shows distinct curvature, indicating biphasic behavior. The respective rate constants for both phases as obtained from a semilogarithmic plot (inset to Figure 6) of the reaction followed under pseudo-first-order conditions with DTNB in excess are 420 and 2100 s^{-1} M^{-1} for the slow and fast phases, respectively. The fast phase rate is about an order of magnitude slower than the corresponding rate between DTNB and the free enzyme.

Discussion

Rate Constants. The advantage of the present approach of measuring the rate constants of irreversible inhibition reactions by following the substrate reaction in the presence of the inhibitor has been pointed out before (Tian & Tsou, 1982; Wang & Tsou, 1987; Tsou, 1988). The conventional method for the determination of the rate constants for the irreversible modification of enzyme activity is to take aliquots from an incubation mixture of the enzyme with the modifier at definite time intervals and assay for the activity remaining. This is not only laborious but also too slow to be applied to fast reactions. As shown for enzymes with a single substrate (Tian & Tsou, 1982; Liu & Tsou, 1986), the rate constants for the reaction between DTNB or iodoacetamide with the enzyme was obtained by the present approach of following the substrate reaction during inactivation for the uncomplexed creatine kinase are of the same order of magnitude with those obtained by the conventional method.

Substrate Competition. Furthermore, the present approach has the added advantage that for enzyme reactions involving two substrates, the modification rate constants between the irreversible inhibitor and the binary and ternary enzyme–substrate complexes can be easily obtained as is illustrated in the present study (Table II). A comparison of these rate constants gives a quantitative measure of the competition of the irreversible inhibitor with the substrates. Some protective effects on both the modification and inactivation reactions of iodoacetamide with the enzyme were observed by Watts and Rabin (1962) in the presence of the equilibrium reaction mixture only but not by the individual substrates separately. In the present study, the presence of substrate seems to have different effects on the inactivation reactions by iodoacetamide and DTNB. For ATP, both inactivators are noncompetitive whereas iodoacetamide competes weakly with creatine, but DTNB does not. Since both reagents react with the active site thiol groups, it does not seem likely that these thiols are involved in creatine binding as suggested by Watts and Rabin (1962). The formation of the ternary complex protects against both DTNB and iodoacetamide inactivation although to different extents. The rate constant for the reaction between iodoacetamide with the ternary complex is severalfold lower than that with the free enzyme, but for DTNB the difference is much greater. This is not surprising as it would be more difficult for the bulkier and negatively charged DTNB to approach the active site when it is fully occupied during the formation of the ternary complex. Alternatively, a conformation change upon the formation of the ternary complex could prevent the reaction with the negatively charged DTNB molecule. The separately determined rate constant between

DTNB and the complex of the enzyme with the transition-state analogue ADP–Mg^{2+}–creatine–NO_3^- is about an order of magnitude lower than that for the reaction with the free enzyme.

Effect of the Transition-State Analogue. Evidence has been accumulating in recent years on the very tight binding of transition-state analogues at the substrate binding sites of enzymes in general. The binding constants are usually several orders of magnitude higher than those of the substrates (Lienhard, 1971; Wolfenden, 1976; Fersht, 1984) indicating that the transition-state analogue mimics the binding of the substrate at the transition state during catalysis involving probably certain conformational changes of the enzyme molecule. The use of ADP–Mg^{2+}–creatine–NO_3^- as a transition-state analogue for creatine kinase in particular was reported by Milner-White and Watts (1971). As the transition-state analogue is extremely tight binding, it can be assumed that at equilibrium it is by far the predominating species bound at the substrate binding site.

Price and Hunter (1976) reported that although the two active site thiols react identically with DTNB or iodoacetic acid for the uncomplexed enzyme, the reaction is biphasic in the presence of the transition-state analogue, suggesting that these two thiols react with different rates while the enzyme is at the transition state during catalysis. Similar results of biphasic kinetics are presented for the DTNB reaction in the presence of the transition-state analogue in the present study at 25 °C and pH 9.0, the optimal pH for the forward reaction. If both of these thiols are essential for the activity of the enzyme and the two subunits are both active independently and if DTNB reacts in the same way with the enzyme in its transition state, the inactivation rate should also be biphasic. Results obtained in the present study show that the inactivation reactions by either DTNB or iodoacetamide as measured in the presence of all the substrates are monophasic reactions. Therefore, it does not seem likely that the minimal active unit of the enzyme is the monomer. Alternatively, if the dimer is the active species and both the reactive thiol groups are essential, the modification of any one of them would lead to the complete inactivation of the enzyme. This again does not seem likely as it has been shown before (Zhou & Tsou, 1987) that the number of essential thiol groups per active unit of this enzyme is one. The above results can be reconciled if of the two reactive thiols (one on each subunit of the dimeric enzyme) only one is essential, the modification of which leads to inactivation, suggesting that the enzyme has to be in the dimeric state to be active.

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Registry No. EC 2.7.3.2, 9001-15-4; DTNB, 69-78-3; Cr, 57-00-1; ATP, 56-65-5; iodoacetamide, 144-48-9.

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