Creatine Kinase. The Relationship of Trypsin Susceptibility to Substrate Binding*

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ABSTRACT: In the presence of Mg^{2+} and at relatively high enzyme concentrations, creatine kinase is digested by trypsin at pH 9.0 at a slow rate, measureable by pH-Stat analysis. Loss of creatine kinase activity is correlated with the extent of digestion. The rate of trypsin digestion of creatine kinase is decreased in the presence of creatine or adenosine 5′-diphosphate (ADP) and the effect of this nonworking substrate pair combined is slightly more than additive. On the assumption that the decline in rate of trypsin digestion is a function of substrate binding, dissociation constants have been calculated for creatine and $MgADP^-$ (110 (±20) and 0.3 (±0.1) mM, respectively) and compared with those determined by other methods. In the presence of a saturating concentration of $MgADP^-$, the dissociation constant for creatine is reduced to 13 (±3) mM. A decrease in the dissociation constant for $MgADP^-$ to 0.07 (±0.03) mM is observed in the presence of high creatine concentration. These results would seem to indicate that the presence of $MgADP^-$ on the enzyme facilitates creatine binding and *vice versa*. Control experiments with other trypsin substrates demonstrated that these effects are not due to interaction of creatine or $MgADP^-$ with trypsin, and therefore, must be a function of their binding to creatine kinase. Adenosine is bound to creatine kinase with a dissociation constant of ~8 mM; under conditions where the enzyme contains about 1.4 moles of adenosine/mole of creatine kinase, the binding of creatine is not increased as compared to the eight- to ninefold increase in the presence of $MgADP^-$.

Cooperative effects in substrate binding to creatine kinase have been observed in several types of studies of this enzyme and have been ascribed typically to conformational changes resulting from the enzyme–substrate interaction. Lui and Cunningham (1966) have shown that creatine kinase is partially protected by both creatine and $MgADP^-$ [1] against digestion by trypsin and that this protection is slightly more than additive. Because only limited insight has been gained from the application of several types of physical methods to the analysis of the postulated conformational change (Samuels, 1961; Lui and Cunningham, 1966; Kägi and Li, 1965), further investigation of the trypsin attack on creatine kinase seemed warranted. These studies have shown that the decrease in rate of trypsin digestion is proportional to the amount of enzyme containing bound substrate. Thus it has been possible to determine dissociation constants for $MgADP^-$ (in the presence and absence of creatine), creatine (in the presence and absence of $MgADP^-$ or adenosine), and for the substrate analog, adenosine.

The results of this investigation indicate that while creatine is relatively loosely bound to the enzyme, the presence of enzyme-bound $MgADP^-$ greatly increases the affinity of the enzyme for creatine. Similarly, the presence of bound creatine causes an increased binding of $MgADP^-$. On the other hand, enzyme-bound adenosine does not increase creatine binding. Although the $MgADP^-$-induced increase in creatine binding may be interpreted in terms of a limited conformational change, other results of this investigation are equally consistent with the possibility that enzyme-bound $MgADP^-$ may form a part of the creatine binding site and in this way be responsible for at least a part of the increase in creatine binding. At the least, it may be concluded that if an ADP-induced conformational change is responsible for increased creatine binding, binding of the adenosine moiety alone does not elicit such a change.

Materials and Methods

Preparation and Assay of Creatine Kinase. Creatine kinase was isolated by method B of Kuby *et al.* (1954) and crystallized two times according to Mahowald *et al.* (1962). Protein concentration was determined by measuring optical density at 280 mμ assuming $E^{1\%}_{1\,cm}$ 8.8 (Noda *et al.*, 1954; Kuby *et al.*, 1962). Specific activity of the crystalline creatine kinase was 50–60 units/mg (Kuby *et al.*, 1954) as determined by the pH-

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[1] Abbreviations used: CrK, creatine kinase; ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; GDP, guanosine 5′-diphosphate; IDP, inosine 5′-diphosphate; Cr, creatine; TAME, *p*-tosyl-L-arginine methyl ester hydrochloride; *p*-NPA, *p*-nitrophenylacetate; BAEE, benzoyl-L-arginine ethyl ester.

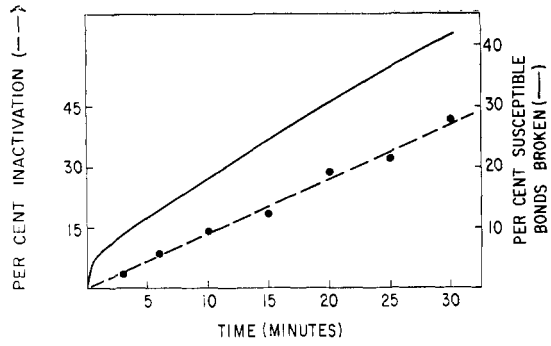

FIGURE 1: Inactivation of creatine kinase during trypsin digestion. The 4-ml trypsin reaction mixture contained 8 mg of CrK, 0.8 mg of trypsin, 1.25×10^{-2} M $MgSO_4$, 8.75×10^{-2} M NaCl, and 1.25×10^{-4} M glycine. At the times shown, 50-μl aliquots were removed for assay of CrK activity (●). The solid line represents per cent of the total susceptible bonds broken as measured by the pH-Stat analysis.

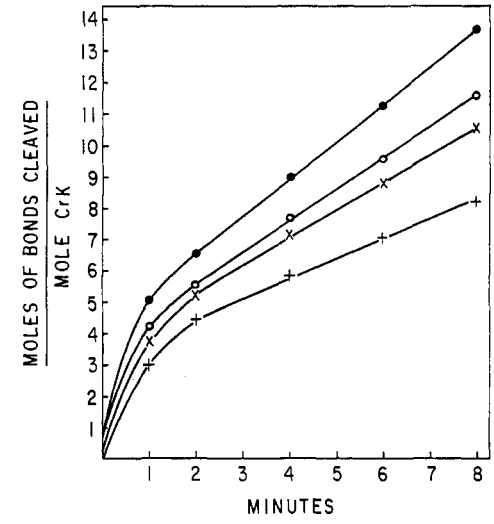

FIGURE 2: Rate of trypsin digestion of CrK alon, (●——●) or in the presence of 0.03 M creatine (○——○) 1.5×10^{-3} M ADP (×——×), or 0.03 M creatine plue 1.5×10^{-3} M ADP (+——+). In addition each 4-ml reaction mixture contained 8 mg of CrK, 0.8 mg of trypsin, 1.25×10^{-2} M $MgSO_4$, 8.75×10^{-2} M NaCl, and 1.25×10^{-4} M glycine.

Stat assay of Mahowald *et al.* (1962).

Trypsin Digestion of Creatine Kinase. Digestion of creatine kinase by trypsin at pH 9.0 and 30° was followed by measuring release of H^+ with a Radiometer pH-Stat titrigraph (Cunningham, 1964) in a nitrogen atmosphere. Each 4-ml reaction mixture contained 1.25×10^{-2} M $MgSO_4$, 8.75×10^{-2} M NaCl, 1.25×10^{-4} M glycine, and 2.47×10^{-5} M creatine kinase. In addition, some reaction mixtures included one or more of the following: ADP (ADP-Li_3, Schwarz BioResearch, Inc.), creatine, glycocyamine (Sigma, technical grade, recrystallized from hot water), and adenosine (Sigma) in varying concentrations. When ADP was present, equimolar quantities of $MgSO_4$ were added in order to maintain the concentration of free Mg^{2+} constant at 1.25×10^{-2} M. Digestion was begun by addition of 50 μl of 0.001 N HCl containing 0.8 mg of trypsin (TRL 6261, Worthington Biochemical Corp.) and the pH was maintained at 9.0 by automatic addition of 0.01 or 0.02 N NaOH.

Control experiments were run to ensure that the effects of substrates on the rate of digestion of creatine kinase were not due to direct inhibitory effects on the trypsin itself. The effects of 0.04 M creatine and 0.04 M glycocyamine were studied using BAEE as substrate for trypsin in a 200-ml reaction mixture containing 0.16 M KCl and 0.01 M $MgSO_4$. When creatine or glycocyamine was absent, the KCl concentration was increased to 0.2 M, although it was found that in this range these small changes in ionic strength had no appreciable effect on the rate of trypsin hydrolysis of BAEE. After an initial equilibration at 30°, each reaction was begun by the addition of 50 μl of 0.001 N HCl containing 5 μg of trypsin. The pH was maintained at 8.2 by automatic addition of 0.01 N NaOH. In another control experiment creatine kinase was replaced (in the basic 4-ml reaction mixture described above) by 7×10^{-5} M ovalbumin denatured according to Nuenke and Cunningham (1961). The digestion of denatured ovalbumin alone was compared with that in the presence of 0.05 M creatine or 1.5×10^{-3} M ADP. Digestion was begun by the addition of 100 μl of 0.001 N HCl containing 0.2 mg of trypsin. Finally, trypsin was also assayed in the presence and absence of 0.06 M creatine or 0.008 M Mg-ADP^- by the method of Rovery *et al.* (1953) using TAME (Mann Research Laboratories, Inc.) as substrate.

Other control experiments were designed to measure autolysis of trypsin during the course of digestion of creatine kinase, both in the presence and absence of substrates. From the basic 4-ml reaction mixture 50-μl aliquots were removed at 0–20 min after addition of trypsin, diluted 40-fold in cold 0.001 N HCl, and assayed immediately for trypsin activity by a spectrophotometric method similar to that of Hummel (1959). Similar assays were made on reaction mixtures containing 0.008 M ADP or 0.06 M creatine or both.

Inactivation of creatine kinase during digestion by trypsin was measured by removing aliquots from a basic 4-ml reaction mixture (containing only $MgSO_4$, NaCl, glycine, creatine kinase, and trypsin) and diluting them 50-fold in cold 0.001 M glycine (pH 9.0). The diluted enzyme was then assayed immediately by the standard pH-Stat procedure (Mahowald *et al.*, 1962).

Results

Inactivation of Creatine Kinase during Trypsin Digestion. When trypsin is added to a solution of creatine kinase, there is a very limited but rapid liberation of hydrogen ions followed by a much slower reaction which

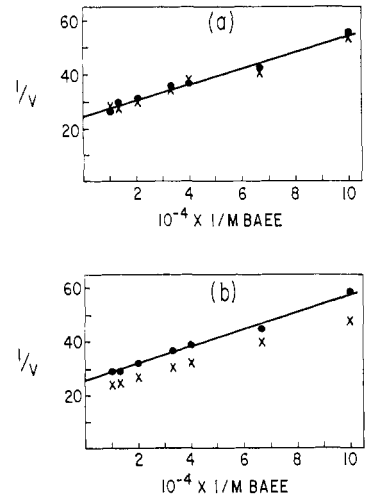

FIGURE 3: Lineweaver–Burk plots of rates of trypsin hydrolysis of BAEE. In addition to BAEE, 0.01 M $MgSO_4$, and 5 μg of trypsin, each 200-ml reaction mixture contained: (a) (●) 0.2 M KCl or (×) 0.16 M KCl and 0.04 M creatine; (b) (●) 0.2 M KCl or (×) 0.16 M KCl and 0.04 M glycocyamine.

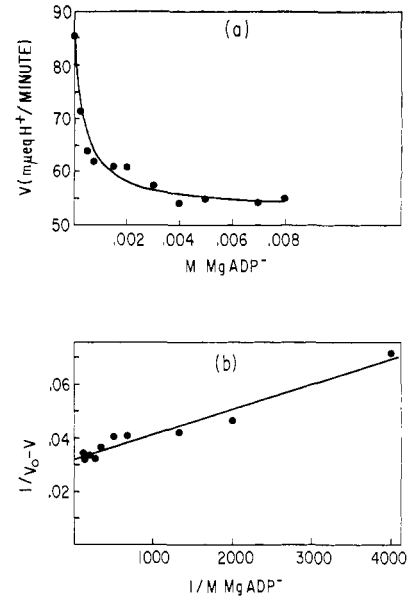

FIGURE 4: A study of trypsin digestion rates. (a) Rate of trypsin digestion (V) of CrK as a function of $MgADP^-$ concentration. In addition to $MgADP^-$ each 4-ml reaction mixture contained 8 mg of CrK, 0.8 mg of trypsin, 1.25×10^{-2} M $MgSO_4$, 8.75×10^{-2} M NaCl, and 1.25×10^{-4} M glycine. (b) Reciprocal plot of decrease in rate of trypsin digestion ($V_0 - V$) *vs.* $MgADP^-$ concentration.

exhibits a relatively constant rate for at least 10 min (Figure 1). The number of bonds broken increases to more than 40% of the total susceptible bonds after 30 min. The "per cent susceptible bonds broken" was calculated on the basis that creatine kinase contains 99 lysine plus arginine residues (Noltmann *et al.*, 1962), and the assumption of an average pK_a' of 8.0 for the α-amino groups released. This pK_a' was estimated by determining the difference in the amount of base necessary to titrate from pH 9.0 to 10.0 before and after digestion of heat-denatured creatine kinase. From these data, the percent of newly released amino groups existing in the charged state at pH 9.0 may be calculated.

Figure 1 also includes the results obtained when aliquots from such a trypsin digestion reaction mixture are assayed for creatine kinase activity after 0–30-min digestion time. Although the experimental error associated with the creatine kinase assay under these conditions is large enough to cause some scatter in the points representing percent inactivation, it may be seen that the rate of inactivation is essentially linear, and that this loss of activity is directly related to the linear phase of the trypsin digestion (see Discussion).

Effects of Added Substrates. We have previously reported that in the presence of Mg^{2+} all substrates of creatine kinase depress the rate at which trypsin attacks this enzyme at pH 9 (Lui and Cunningham, 1966). Although the kinetics of this reaction are not clearly understood, the slope of the linear portion can be used to compare the susceptibility of the enzyme to trypsin digestion under various conditions. In Figure 2 it can be seen that in the presence of 1.25×10^{-2} M Mg^{2+}, 3×10^{-2} M creatine and 1.5×10^{-3} M ADP decrease the rate of trypsin attack by about 17 and 27%, respectively. Together they depress the rate by about 50%.

Control experiments, using denatured ovalbumin or TAME as substrate for trypsin, indicated that the presence of creatine, ADP, or a combination of the two, had no effect on the activity of the trypsin. However, the probability that these substrates have a much smaller K_m (with respect to trypsin) than does native creatine kinase led us to check this conclusion further in a system where the substrate concentration could be varied over a range optimal for the detection of minimal inhibition of trypsin activity by creatine. Figure 3a shows a Lineweaver–Burk plot of the kinetic data obtained when BAEE is used as trypsin substrate in the presence of 0.04 M creatine. Under the conditions used, a K_i as large as 100 mM should be detectable. However, no inhibition was observed as indicated in Figure 3a where all the points may be seen to fall on a common line both in the presence and absence of creatine.

It should be noted that although there are 99 potentially trypsin-susceptible bonds (lysine plus arginine) (Noltmann *et al.*, 1962) in creatine kinase, only about 20% of these bonds are hydrolyzed during the period in which our rate measurements were made. Since this limited attack on creatine kinase by rather high concentrations of trypsin was significantly inhibited by the presence of substrates of the kinase, it seemed likely that trypsin susceptibility might be directly correlated with substrate binding at the active site.

Figures 4a, 5a, and 6a indicate the results when the rate of trypsin digestion was studied as a function of the

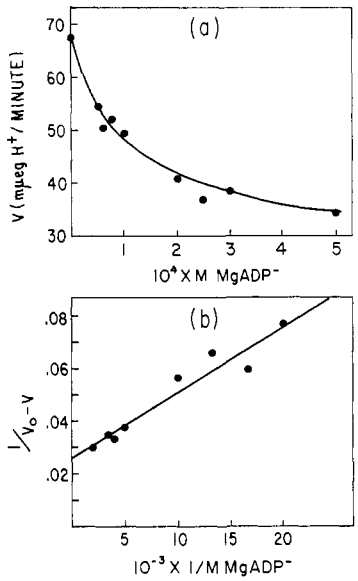

FIGURE 5: A study of trypsin digestion rates. (a) Rate of trypsin digestion (V) of CrK as a function of $MgADP^-$ concentration in the presence of 0.06 M creatine. In addition each 4-ml reaction mixture contained 8 mg of CrK, 0.8 mg of trypsin, 1.25×10^{-2} M $MgSO_4$, 8.75×10^{-2} M NaCl, and 1.25×10^{-4} M glycine. (b) Reciprocal plot of decrease in rate of trypsin digestion ($V_0 - V$) *vs.* $MgADP^-$ concentration.

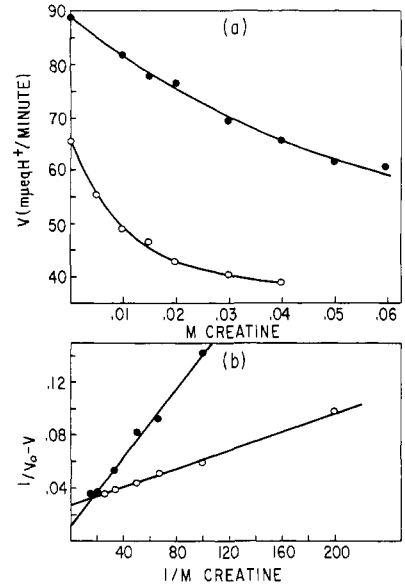

FIGURE 6: A study of trypsin digestion rates. (a) The rate of trypsin digestion (V) of CrK as a function of creatine concentration, alone (●——●) or in the presence of 8×10^{-3} M $MgADP^-$ (○——○). In addition each 4-ml reaction mixture contained 8 mg of CrK, 0.8 mg of trypsin, 1.25×10^{-2} M $MgSO_4$, 8.75×10^{-2} M NaCl, and 1.25×10^{-4} M glycine. (b) Reciprocal plots of decrease in rate of trypsin digestion ($V_0 - V$) *vs.* creatine concentration, alone (●——●) or in the presence of 8×10^{-3} M $MgADP^-$ (○——○).

concentration of $MgADP^-$, creatine, or one of these substrates in the presence of the other. Figure 4a shows the decrease in rate of digestion of creatine kinase as the concentration of $MgADP^-$ is increased from 0 to 0.008 M. A similar, though less marked, decrease is seen in Figure 6a as creatine concentration is increased over the range of 0–0.06 M. Figure 6a also shows the decreased rate of trypsin digestion as creatine is increased to 0.04 M in the presence of a constant saturating concentration of $MgADP^-$ and an analogous decrease is seen in Figure 5a when $MgADP^-$ concentration is increased in the presence of 0.06 M creatine.

If the formation of the enzyme–substrate complex is described by the equation

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES$$

then

$$\frac{d(ES)}{dt} = k_1(E)(S) - k_2(ES) \qquad (1)$$

where (E) = concentration of free enzyme. At equilibrium $d(ES)/dt = 0$ so that

$$(E) = \frac{k_2(ES)}{k_1(S)} = K_D\frac{(ES)}{(S)} \qquad (2)$$

Since (E) = (E_t) − (ES), where (E_t) = total concentration of enzyme

$$(ES) = \frac{(E_t)(S)}{K_D} - \frac{(ES)(S)}{K_D}$$

Rearranging this equation

$$\frac{1}{(ES)} = \frac{K_D}{(E_t)(S)}\frac{1}{} + \frac{1}{(E_t)} \qquad (3)$$

If the decrease in rate of trypsin digestion ($V_0 - V$) is proportional to the concentration of enzyme-bound substrate, then

$$\frac{1}{V_0 - V} = \frac{K_D}{V_0 - V_{min}(S)}\frac{1}{} + \frac{1}{V_0 - V_{min}} \qquad (4)$$

where V_0 = rate of trypsin digestion when (S) = 0, V = rate of trypsin digestion at other substrate concentrations, V_{min} = rate of trypsin digestion when (S) = ∞, and $V_0 - V_{min}$ = maximum decrease in rate of trypsin digestion when (S) = ∞.

In the case of creatine alone

$$K_{D_{Ia}} = \frac{(CrK)(Cr)}{(CrK\text{–}Cr)} \qquad (5)$$

and analogously for the case of $MgADP^-$ alone

$$K_{D_{1b}} = \frac{(CrK)(MgADP^-)}{(CrK\text{–}MgADP)} \quad (6)$$

For creatine in the presence of $MgADP^-$

$$K_{D_{2a}} = \frac{(CrK\text{–}MgADP)(Cr)}{(CrK\text{–}MgADP\text{–}Cr)} \quad (7)$$

and for $MgADP^-$ in the presence of creatine

$$K_{D_{2b}} = \frac{(CrK\text{–}Cr)(MgADP^-)}{(CrK\text{–}Cr\text{–}MgADP)} \quad (8)$$

Figures 4b, 5b, and 6b show reciprocal plots (eq 4) for the data obtained from Figures 4a, 5a, and 6a, respectively. Values for K_D have been calculated from the intercepts and the slopes of such plots. The dissociation constant for creatine from creatine kinase was 110 (±20) mM. In the presence of $MgADP^-$, the dissociation constant was reduced to a value of 13 (±3) mM, indicating that bound $MgADP^-$ effects an eight- to ninefold increase in the binding of creatine to creatine kinase. The dissociation constant for $MgADP^-$ was found to be 0.3 (±0.1) mM. When 0.06 M creatine is added to the system, creatine kinase should bind at least 0.7 mole of creatine/mole of enzyme ($K_{D_{1a}}$ = 110 mM). Under these conditions the dissociation constant for $MgADP^-$ is significantly decreased to 0.07 (±0.03) mM. Presumably this value would be even smaller if it were technically possible to saturate the enzyme with creatine. This determination of K_D for $MgADP^-$ in the presence of creatine is further complicated by the fact that more creatine will be bound as the concentration of ADP is increased.

In order to ensure a constant level of 1.25×10^{-2} M free Mg^{2+} in all solutions, additional and equimolar quantities of $MgSO_4$ were added along with ADP to all reaction mixtures. This was necessary because the rate of digestion was found to exhibit a significant dependence on the concentration of free Mg^{2+}. ADP is known to bind Mg^{2+} with a stability constant of at least 1×10^{-3} M^{-1} (Kuby *et al.*, 1962; O'Sullivan and Perrin, 1964), so that essentially all of the ADP present is in the $MgADP^-$ form at the concentrations employed in these experiments.

Effects of Substrate Analogs on the Rate of Trypsin Digestion of Creatine Kinase. In view of the fact that both creatine and $MgADP^-$ decrease the susceptibility of creatine kinase to trypsin digestion, it was of interest to investigate the effects of analogs of these two substrates. Since glycocyamine can act weakly as a substrate for creatine kinase (Tanzer and Gilvarg, 1959), it might be expected to bind at least slightly to the enzyme. However, comparison of glycocyamine and creatine in the trypsin–BAEE system (Figure 3b) shows that glycocyamine is a poor choice for study in the trypsin–creatine kinase system, since it apparently interacts with trypsin, stimulating the rate of hydrolysis of BAEE. This conclusion was borne out in the trypsin–creatine kinase system when an apparent K_D of 60 mM for the glycocyamine–creatine kinase complex (alone or in the presence of MgADP) was calculated from the trypsin digestion data. Although it is, of course, conceivable that glycocyamine might bind to creatine kinase better than creatine in the absence of ADP, kinetic studies varying creatine in a creatine kinase assay reaction mixture containing 0.038 M glycocyamine indicated a very large K_I (>200 mM) for glycocyamine, a value which is inconsistent with the K_D determined by trypsin digestion. Thus, as Figure 3b indicates, an interaction of glycocyamine with trypsin probably accounts for the reduction in the rate of attack of the protease on creatine kinase.

Attempts were made to test the effects of purine ribotide analogs of ADP, such as GDP, IDP, AMP, and adenosine. Unfortunately, GDP and IDP both have functional groups with pK's in the range of 9.0–9.6, so that the buffering effects of these two analogs have made it impossible so far to obtain useful trypsin digestion data by pH-Stat analysis at pH 9.0. The stability constant of the MgAMP complex is so low that it is impossible to simultaneously maintain (1) most of the AMP in the MgAMP form and (2) a constant concentration of free Mg^{2+}, without using extremely high Mg^{2+} concentrations. However, it was possible to determine a dissociation constant of 8 (±3) mM for adenosine in this system, although the limited solubility of this compound did present some difficulties. With this value it can be shown that at 0.02 M adenosine, creatine kinase should be almost 75% saturated with adenosine. Figure 7a shows the effect of creatine on the rate of trypsin digestion in the presence of 0.02 M adenosine and Figure 7b shows a reciprocal plot from which the creatine dissociation constant was calculated to be 96 (±20) mM. This value does not differ significantly from that of 110 (±20) mM obtained for creatine alone, indicating that enzyme-bound adenosine does not significantly increase creatine binding in the manner demonstrated for ADP.

Effects of Trypsin Autolysis. During the course of the work described above, it was found that trypsin undergoes a small amount of autolysis (less than 15%) under the conditions of these experiments, even in the presence of Mg^{2+}. Since all experiments were done under identical conditions (except for addition of various substrates or analogs) and all rates of digestion of creatine kinase were obtained from the linear portion of the curve between 4 and 12 min, a small amount of trypsin inactivation during this time would not be expected to appreciably affect the relative values for digestion rates at different substrate concentrations. However, it was of interest to examine the effect of creatine and ADP on the autolysis rate of trypsin. In the basic reaction mixture (containing $MgSO_4$, NaCl, glycine, and creatine kinase) trypsin had lost 14% of its activity at the end of 12 min. The addition of 0.06 M creatine, the highest concentration we have used, resulted in an 11% activity loss during the same time period while the addition of 0.008 M ADP (in

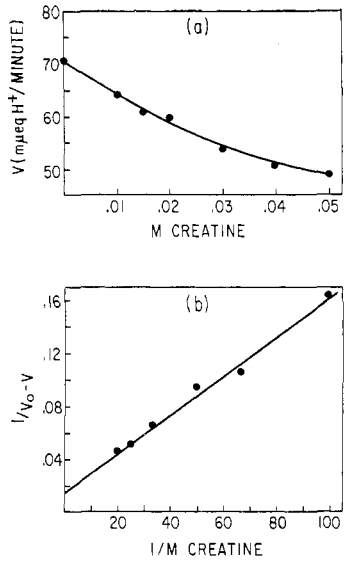

FIGURE 7: A study of trypsin digestion rates. (a) Rate of trypsin digestion (V) of CrK as a function of creatine concentration in the presence of 2×10^{-2} M adenosine. In addition each 4-ml reaction mixture contained 8 mg of CrK, 0.8 mg of trypsin, 1.25×10^{-2} M $MgSO_4$, 8.75×10^{-2} M NaCl, and 1.25×10^{-4} M glycine. (b) Reciprocal plot of decrease in rate of trypsin digestion ($V_0 - V$) *vs.* creatine concentration in the presence of 2×10^{-2} M adenosine.

the presence or absence of 0.06 M creatine) made little if any change in the values stated above. During the limited interval (4–12 min) used to estimate the rate of hydrolysis, the decrease in trypsin activity was about 7 and 9% in the presence and absence of creatine, respectively. The effects of this limited trypsin inactivation upon the calculation of dissociation constants are thus well within the experimental error of this system. This evaluation is clearly borne out by the comparison, where possible, of dissociation constants evaluated by the trypsin digestion technique with those obtained by equilibrium dialysis or kinetic analyses.

Discussion

The muscle enzyme, creatine kinase, is almost completely specific for its substrates, $MgATP^{2-}$ and creatine. No analogs of creatine have been shown to participate in the transphosphorylating reaction except glycocyamine (Tanzer and Gilvarg, 1959) and *N*-ethylglycocyamine (Ennor *et al.*, 1955) and these at very low rates compared to creatine. This narrow specificity coupled with the relatively weak binding of creatine (K_m = 19 mM; Nihei *et al.*, 1961) evident from kinetic studies led us to investigate details of this creatine–creatine kinase interaction, particularly how it is affected by the simultaneous presence of a nucleotide substrate. Evidence for cooperative effects in substrate binding had been obtained by a variety of techniques including changes in immunological properties (Samuels, 1961), variations in reactivity of the sulfhydryl group in the active site (O'Sullivan *et al.*, 1966), and kinetic analysis of the over-all catalytic reaction (Morrison and James, 1965). These cooperative effects were uniformly interpreted as indicating conformational changes in the enzyme, although two recent attempts to detect conformational change by the physical techniques of optical rotatory dispersion (Kägi and Li, 1965) and deuterium exchange (Lui and Cunningham, 1966) have not shown measurable effects to result from the simultaneous presence of both substrates. Thus, conformational change would appear to be restricted to relatively small movements in the immediate vicinity of the active site, perhaps involving only concerted displacements of critical side chains. Experimental probes which might detect such localized changes are rather limited, but one, sensitivity to trypsin hydrolysis, seemed suited to the study of creatine kinase.

Although this enzyme contains 99 lysine plus arginine residues per mole (81,000) it is markedly stable toward trypsin, undergoing only slow proteolysis at CrK: trypsin ratios of 10:1. The inactivation of creatine kinase by trypsin is directly related to the per cent of susceptible bonds which have undergone proteolysis (Figure 1). Although the curve which represents the rate of peptide bond hydrolysis is biphasic, the rate of inactivation appears to be essentially linear. One interpretation of this behavior is that the brief period of rapid initial hydrolysis may reflect cleavage of the polypeptide chain in a region remote from the active site, where its integrity is not essential to enzymatic activity. A more likely interpretation is that these data simply indicate the presence in the sample of a small quantity of denatured creatine kinase (less than 4%), resulting from dialysis and freeze-drying, which is rapidly and completely attacked at this high trypsin concentration. In any event the subsequent slower and prolonged attack by trypsin on the enzyme is clearly related to loss of enzymatic activity. In preliminary ultracentrifuge studies of the trypsin digestion of creatine kinase (terminated at various intervals by addition of soybean trypsin inhibitor), no fragments of intermediate size could be detected. These results suggest that after the initial attack of trypsin on a creatine kinase molecule the remainder of that molecule is degraded very quickly, so that the digestion is probably an all or none reaction.

Although the rate of trypsin digestion is nearly identical with the rate of inactivation, there is no reason to suppose that the two should be exactly the same since some of the potentially susceptible bonds in creatine kinase may be wholly or partially masked. If one assumes that less than 99 bonds are actually susceptible to trypsin under the conditions of hydrolysis employed, then the rate of bonds broken (on a percentage basis) should be slightly higher than that shown in Figure 1. In fact, a small correction of the rate of digestion in this direction could make it identical with the rate of inactivation (1.4%/min). However, in view of the magnitude of the errors involved in assay-

ing creatine kinase, it is doubtful whether such a correction would be meaningful.

Since tryptic hydrolysis requires at least the momentary availability of lysine or arginine residues, changes in the susceptibility to trypsin or other proteolytic enzymes in the presence of substrates have sometimes been taken as indicating the general conformational stability of a protein (Markus, 1965; Elödi and Szabolcsi, 1959; Taketa and Pogell, 1965). The low susceptibility of CrK toward trypsin is further reduced when either or both of the substrates are bound to the active site. It may be postulated that this decrease in susceptibility results from: (1) stabilization of a more tightly folded conformation of the enzyme, (2) localized conformational changes involving lysine or arginine residues, or (3) a direct masking of a susceptible lysine or arginine residue in the active site. The relative contribution of these possibilities to the creatine kinase system cannot as yet be distinguished. It may be recalled, however, that neither optical rotatory dispersion (Kägi and Li, 1965) nor deuterium-exchange studies (Lui and Cunningham, 1966) could detect conformational change as a result of creatine binding to the $MgADP^-$–CrK complex, although this does result in additional protection against trypsin and in the appearance of a marked protection against inhibition by both iodoacetamide and *p*-nitrophenyl acetate (Lui and Cunningham, 1966). Furthermore, evidence has been presented for the participation of a trypsin susceptible residue, lysine, in the catalytic mechanism of the enzyme (Watts, 1963; Clark and Cunningham, 1965; Jacobs and Cunningham, 1966).

Further insight into the structure of these creatine kinase–substrate complexes has been made possible by the study of the dependence of trypsin susceptibility upon creatine concentrations. It was first necessary, however, to show that the effects of creatine and ADP in decreasing the rate of trypsin digestion were not due to inhibition of the trypsin itself. Trypsin activity, with 10^{-4}–10^{-5} M BAEE as substrate, did not vary with the addition of 0.04 M creatine. In the presence of high concentrations of creatine and/or ADP the rates of trypsin hydrolysis of both denatured ovalbumin and TAME were identical, within experimental limits, with those found in the absence of these compounds. Under the same experimental conditions, inhibition of trypsin by 0.05 M benzamidine (Diniz *et al.*, 1965) was easily detected.

If it is assumed that the linear rate of trypsin digestion is highest for free enzyme and is reduced proportionally as the percentage of enzyme containing bound substrates increases, it is possible to calculate dissociation constants for creatine from the CrK–creatine complex, $K_{D_{1a}} = 110\ (\pm 20)$ mM, and from the CrK-$MgADP^-$–Cr complex, $K_{D_{2a}} = 13\ (\pm 3)$ mM. Early attempts to measure creatine binding by equilibrium dialysis yielded only a minimum estimate of the dissociation constant for the CrK–Cr complex, ≥ 10 mM (Kuby *et al.*, 1962). More recently, kinetic analysis has determined an analogous $K_{D_{1a}}$ of 16–19 mM and $K_{D_{2a}}$ of 8–18 mM (Morrison and James, 1965). However, O'Sullivan *et al.* (1966) determined dissociation constants of 50 and 10 mM in the absence and presence of $MgADP^-$, respectively, by titrating the protective effect of creatine against iodoacetate inhibition.

Values reported for the dissociation constant of the $MgADP^-$–CrK complex have ranged from 0.05 to 0.17 mM in kinetic studies of the enzyme-catalyzed reaction (James and Morrison, 1966; Morrison and James, 1965) and by equilibrium dialysis measurements (Kuby *et al.*, 1962). The kinetic analysis of Morrison and James (1965) showed only a small decrease in the dissociation constant of the $MgADP^-$–CrK complex in the added presence of creatine. The values we obtained from trypsin digestion rate studies were 0.07 (± 0.03) and 0.3 (± 0.1) mM in the presence and absence of creatine, respectively. Since it was impossible to saturate the enzyme with creatine, the value of 0.07 (± 0.03) mM must be considered only as an approximate dissociation constant; however, these data would seem to indicate that enzyme-bound creatine facilitates the binding of $MgADP^-$ in much the same way that the reverse situation occurs.

The agreement between the constants obtained by the trypsin digestion method and those determined by other techniques seems acceptable in view of the wide differences in the experimental and theoretical bases of these measurements and would seem to support a direct relationship between trypsin susceptibility and substrate binding at the active site. A similar conclusion has been drawn for hexokinase where glucose or fructose was used to protect this enzyme against tryptic hydrolysis, and dissociation constants were calculated for these two substrates (Trayser and Colowick, 1961).

Of perhaps greatest importance is the observation that the binding of creatine to CrK is greatly increased in the presence of bound $MgADP^-$. It is of interest to note that isotope-exchange studies (Morrison and Cleland, 1966) have confirmed the formation of an enzyme–MgADP–creatine dead-end complex, which had been suggested earlier by the kinetic studies of Morrison and James (1965). Potentiation of creatine binding may imply (1) that a limited conformational change induced by $MgADP^-$ binding (Lui and Cunningham, 1966) exposes or activates an additional component of the creatine site or (2) that the bound metal–nucleotide complex itself interacts directly with creatine. The first possibility has been described for several enzymes, notably by Koshland (1962, 1964) for phosphoglucomutase and actin. The second possibility is perhaps best illustrated by the hypothetical complex of Figure 8. Here is pictured a substrate complex, showing the association of creatine with both the β and α phosphates of ATP. Creatine binding, potentiated by the interaction at the β phosphate of enzyme-bound $MgADP^-$, then might be responsible for the observed protection against trypsin, iodoacetamide, and *p*-NPA in the presence of the nonworking pair, $MgADP^-$ and creatine. In addition, the analogous potentiation of $MgADP^-$ binding by creatine is easily accounted for in this scheme. The complex pictured in Figure 8

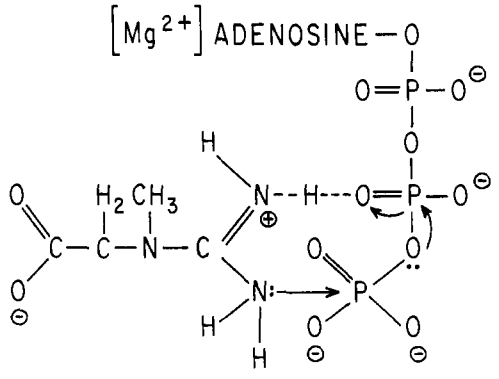

FIGURE 8: Suggested enzyme-bound substrate complex.

has the additional attribute that the electron shifts indicated by the arrows could account for the reaction catalyzed by the enzyme, reversible phosphate exchange from $MgATP^{2-}$ to creatine without a phosphoenzyme intermediate.

It is possible, of course, that both conformational changes and direct nucleotide–creatine interactions occur in this system. Kinetic analyses of the transphosphorylation reaction have indicated that the binding of one substrate to creatine kinase enhances the binding of the second substrate provided the two substrates formed a working pair; however, this type of analysis was unable to detect an analogous facilitation of binding in the case of the nonworking substrate pair, $MgADP^-$ and creatine (Morrison and James, 1965; James and Morrison, 1966). O'Sullivan *et al.* (1966) have found that the reactivity of the active-site sulfhydryl group to iodoacetic acid is increased in the presence of bound $MgADP^-$, while the $MgADP^-$–creatine pair offers protection against iodoacetate inactivation. Furthermore, the dissociation constant for creatine was decreased fivefold in the presence of bound $MgADP^-$. These observations were interpreted in terms of conformational changes in the protein, induced by substrate binding.

The dissociation constant for adenosine, determined at pH 9 by trypsin digestion is 8 (±3) mM. This value is comparable to the K_i of 7 mM determined kinetically at pH 9.0 by Noda *et al.* (1960); however, it varies significantly from the dissociation constant of 49.7 (±22.0) mM determined kinetically at pH 8.0 by James and Morrison (1966). Limited solubility of adenosine prevented its use in concentrations which would guarantee complete saturation of creatine kinase, but if a dissociation constant of 8 mM is assumed, creatine kinase should contain almost 1.5 moles of bound adenosine/mole at 0.02 M adenosine. At this degree of saturation, one would expect that if binding of the adenosine moiety produces a minimal conformational change in creatine kinase similar to that which may be elicited by $MgADP^-$ (or $MgATP^{2-}$), exposing all or part of a specific creatine binding site, then the binding of creatine should be enhanced at least 75% as much as in the case where the enzyme is saturated with $MgADP^-$. In fact, the dissociation constant for creatine in the presence of 0.02 M adenosine is 96 ± 20 mM, not significantly different from that found in the absence of bound nucleoside. The failure of bound adenosine to promote creatine binding cannot be interpreted as ruling out conformational change as an explanation for these cooperative phenomena, but it would appear to further restrict the extent and magnitude of such changes within the active site itself, presumably to side-chain interactions with the pyrophosphate moiety. There is, of course, no possibility for a direct adenosine–creatine interaction such as that postulated in Figure 8 for enzyme-bound $MgADP^-$ and creatine.

It is interesting that glycocyamine, differing from creatine only by the absence of a methyl group, is more weakly bound to creatine kinase, while apparently the reverse is true for the binding of these compounds to trypsin. This is, furthermore, an unfortunate circumstance which has precluded our detecting possible potentiation of glycocyamine binding to creatine kinase in the presence of $MgADP^-$. Such information would be most helpful in the analysis of the binding and mechanism of action of this enzyme, and further studies involving other physical techniques are in progress.

The observations which have been made, however, are consistent with a model in which either creatine or $MgADP^-$ may form a part of the binding site for the other, the presence of either of these substrates increasing the affinity of the enzyme for the other. These observations do not rule out the possibility of facilitated binding due to conformational change; however, failure to detect any such change by optical rotatory dispersion or deuterium exchange due to the presence of creatine would appear to limit the possibilities to small, localized changes in the active site of the enzyme.

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