An Essential Arginyl Residue at the Nucleotide Binding Site of Creatine Kinase†

C. L. Borders, Jr.,‡ and James F. Riordan*

ABSTRACT: Treatment of rabbit muscle creatine kinase (EC 2.7.3.2) with either butanedione in borate buffer or phenylglyoxal in Veronal buffer decreases enzymatic activity correlating with the modification of a single arginyl residue per subunit of the dimeric enzyme. Very little activity is lost when modification is performed in the presence of MgATP or MgADP. Nucleotide binding to the modified enzyme is virtually abolished as determined by ultraviolet difference spectroscopy. The data suggest that an arginyl residue plays an essential role in the enzymatic mechanism of creatine kinase, probably as a recognition site for the negatively charged oligophosphate moiety of the nucleotide.

Recent reports from this laboratory have suggested a general role for arginyl residues in the functional binding of anionic substrates or cofactors to enzymes. A single arginine participates in binding the terminal carboxyl group of peptide substrates to carboxypeptidase A (Riordan, 1970, 1973), and arginyl residues are essential for the binding of substrates to *Escherichia coli* alkaline phosphatase (Daemen and Riordan, 1974). Arginine is also essential for NADH binding of the alcohol dehydrogenases from human liver, horse liver, and yeast (Lange et al., 1974). We have recently demonstrated that an arginyl residue is involved in binding the C-1 phosphate of substrates in rabbit muscle fructose-1,6-bisphosphate aldolase (Lobb et al., 1975). Further, arginyl residues are essential at nucleotide recognition sites in two different ATP-dependent synthetases, glutamine synthetase and carbamoyl-phosphate synthetase (Powers and Riordan, 1975).

Thus far the identity of ATP-binding sites in enzymes that transfer phosphorus-containing groups, i.e., the kinases, has essentially remained unknown. The present study was initiated to determine if arginyl residues might serve as the positively charged receptor sites for binding MgADP and MgATP to creatine kinase (EC 2.7.3.2). Arginine-specific α-dicarbonyl reagents rapidly inactivate the enzyme, and loss of activity correlates with the modification of one arginine per subunit. Protection from inactivation by magnesium nucleotides and loss of nucleotide binding to the modified enzyme implicate an arginyl residue at the nucleotide binding site of creatine kinase. These observations extend the demonstration that arginines participate in the recognition of anionic substrates and cofactors and suggest that they play the same role in other kinases as well as other nucleotide-utilizing enzymes. A preliminary report of this work has appeared (Borders and Riordan, 1975).

Materials and Methods

Creatine hydrate, creatine phosphate, ADP, ATP, dithiothreitol, and 5,5′-dithiobis(2-nitrobenzoic acid) were obtained from Sigma. Phenylglyoxal monohydrate and 2,3-butanedione were products of Aldrich and were used without further purification. Bio-Gel P-4 was obtained from Bio-Rad and Sephadex G-200 and G-25 were obtained from Pharmacia. Magnesium acetate tetrahydrate was a product of Fisher and was used as a source of Mg^{2+} in all assays and experiments to avoid interference by other counterions (Watts, 1973). Acetophenone-7-^{14}C was obtained from ICN, and was used to prepare phenylglyoxal-7-^{14}C, with a specific activity of 147,000 cpm/μmol, by the method of Riley and Gray (1943). All other chemicals were reagent grade.

Rabbit muscle creatine kinase was purchased from Sigma (70–75 activity units/mg) and Boehringer-Mannheim (85–90 units/mg). Both preparations exhibited minor contaminants by disc gel electrophoresis (Davis, 1964). For experiments involving amino acid analysis and/or ^{14}C incorporation, the Boehringer-Mannheim preparation was further purified by gel filtration on a Sephadex G-200 column (2.5 × 90 cm) using 0.1 *M* Tris (pH 7.5) as eluent. This preparation gave a single band on disc gel electrophoresis, an amino acid analysis consistent with published values (Yue et al., 1967), and a specific activity of 110 units/mg.

Enzymatic activity was determined at 30° by the pH-Stat method (Mahowald et al., 1962), and expressed as mequiv of NaOH/min. Protein concentrations were determined from absorbance at 280 nm, using $A_{1cm}(1\%)$ 8.8 (Noda et al., 1954) and a molecular weight of 82,600 (Yue et al., 1967).

Chemical modification reactions were carried out under conditions given in the figure and table legends. All reactions were performed at 25° and were initiated by adding an aliquot of a freshly prepared stock solution of modifying agent in the appropriate buffer to a solution of the enzyme, in some cases together with substrates, in the same buffer. Aliquots were withdrawn, assayed, and compared to a control. It was found during the course of these experiments that the age of the phenylglyoxal stock solutions, varying from a few minutes to 10 days, had no discernible effect on the rate of inactivation of creatine kinase.

The reversibility of butanedione inactivation on the removal of borate was determined by first inactivating the enzyme with 0.23 m*M* butanedione in 50 m*M* borate (pH 8.7). The activity was decreased to 10% of the control after

† From the Biophysics Research Laboratory, Department of Biological Chemistry, Harvard Medical School, and the Division of Medical Biology, Peter Bent Brigham Hospital, Boston, Massachusetts 02115. *Received May 20, 1975.* This work was supported by Grant-in-Aid GM-15003 from the National Institutes of Health, of the Department of Health, Education and Welfare.

‡ On research leave from the Department of Chemistry, College of Wooster, Wooster, Ohio.

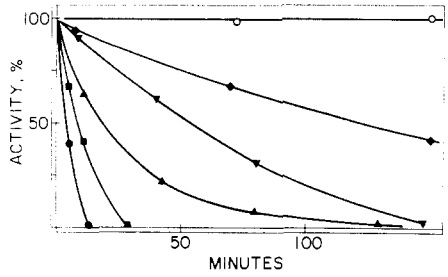

FIGURE 1: Changes in the activity of creatine kinase, 3 μM, in 50 mM borate (pH 8.7) vs. time as a function of butanedione concentration: 0.06 mM (♦), 0.11 mM (▼), 0.23 mM (▲), 0.57 mM (■), and 1.14 mM (●). The native enzyme (○) retains full activity over this period of time.

80 min, at which time aliquots were subjected to gel filtration over a Bio-Gel P-4 column (0.9 × 21 cm), equilibrated with either 50 mM borate or 50 mM Tris (pH 8.7), to remove excess reagent. Aliquots of each eluate containing the modified enzyme were subsequently assayed at various times over a period of 6 hr and compared to a control to determine the extent of reactivation.

The modification of specific amino acid residues by butanedione was determined by amino acid analysis after acid hydrolysis. Aliquots of the reaction mixture were separated from excess reagent by gel filtration over a Bio-Gel P-4 column (0.9 × 21 cm), equilibrated with 50 mM borate (pH 8.7). Fractions containing protein were immediately assayed for enzymatic activity, and aliquots were hydrolyzed in evacuated, sealed ampoules with 6 N HCl at 110° for 18–24 hr. Amino acid analyses were performed using either a Beckman Spinco 120C or a Durrum D-500 amino acid analyzer. Modification of arginine and potential modification of lysine were determined by using a value of 17 His/subunit (Yue et al., 1967) as the basis of reference. Unmodified creatine kinase gave values of 18.0 Arg/subunit and 34.1 Lys/subunit for the average of five analyses, which compares favorably with values of 17.5 and 33.1, respectively, as reported by Yue et al. (1967).

The binding of MgADP and MgATP to native and butanedione-modified creatine kinase was determined by ultraviolet difference absorption spectroscopy after the method of Roustan et al. (1968). Modified creatine kinase was prepared by reacting the enzyme (40 μM) with 0.23 mM butanedione in 50 mM borate (pH 8.7). After 3 hr, aliquots were assayed for enzymatic activity, additional aliquots were subjected to difference spectroscopy in the presence of 0.11 mM MgADP or 0.32 mM MgATP, and a third aliquot was subjected to acid hydrolysis and amino acid analysis.

Modification with [^{14}C]phenylglyoxal in 100 mM bicarbonate (pH 8.7) was monitored by ^{14}C incorporation and amino acid analysis. Aliquots of the reaction mixture were separated from excess reagent by Bio-Gel P-4 chromatography using 100 mM bicarbonate (pH 8.7) as eluent. Protein fractions were immediately assayed for enzymatic activity. Aliquots were also diluted into 10 ml of Aquafluor (New England Nuclear), and radioactivity was determined using a Beckman LS-250 liquid scintillation counter. Additional aliquots were subjected to acid hydrolysis and amino acid analysis.

The reactive sulfhydryl groups of native and modified creatine kinase were titrated with 5,5′-dithiobis(2-nitrobenzoic acid) on a Cary 14 spectrophotometer by a modification of the method of O'Sullivan (1971), using a molar absorptivity at 412 nm of 1.36 × 10^4 for the nitrothiophenolate anion (Ellman, 1959). Preliminary experiments indicated the same 412-nm absorbance for the nitrothiophenolate anion at pH 8.0 and 8.7. Titrations were routinely performed at the latter pH in either 50 mM borate or 100 mM bicarbonate, the conditions for arginine modification. Neither butanedione nor phenylglyoxal, in the concentrations normally used for modification, caused the release of the nitrothiophenolate anion. In a typical modification reaction with either butanedione or phenylglyoxal, aliquots were assayed periodically for enzymatic activity and larger aliquots were subjected to titration with 5,5′-dithiobis(2-nitrobenzoic acid).

Creatine kinase in which the two reactive sulfhydryl groups were reversibly blocked by mixed disulfide formation in the presence of 5,5′-dithiobis(2-nitrobenzoic acid) was prepared by the method of O'Sullivan (1971). Excess reagent was removed by gel filtration on a column (0.9 × 25 cm) of Sephadex G-25 in the presence of 50 mM borate (pH 8.7). Such preparations exhibited less than 0.5% the enzymatic activity of the unmodified enzyme, but could be restored to full activity by dilution into dithiothreitol in 50 mM borate (pH 8.7), the final dithiothreitol concentration being 6 mM. The mixed disulfide of creatine kinase thus prepared was reacted with 0.23 mM butanedione in 50 mM borate (pH 8.7) and at various times aliquots were withdrawn, diluted into a final concentration of 6 mM dithiothreitol–50 mM borate (pH 8.7), and assayed for enzymatic activity 5 min after dilution.

Table I: Effect of pH on the Inactivation of Creatine Kinase by Butanedione.[a]

pH	Activity (%)[b]	$t_{1/2}$ (min)[c]
8.7	34	19
7.5	76	87
6.3	94	320

[a] Creatine kinase, 3 μM, was modified at 25° by 0.23 mM butanedione in 50 mM borate at the indicated pH. Aliquots were assayed at various time intervals and compared to the native enzyme as a control. [b] Measured after 30-min reaction. [c] Determined from a semilog plot of residual activity vs. time.

Table II: Creatine Kinase Inactivation by Butanedione and the Effect of Borate.[a]

[Borate] (mM)	Activity (%)	[Borate] (mM)	Activity (%)
0[b]	95	125	36
4	18	250	72
20	6	500	89
50	6		

[a] Creatine kinase, 3 μM, was modified by 0.23 mM butanedione in borate buffer (pH 8.7), 25°, for 80 min. Aliquots were assayed and compared to the native enzyme as a control. [b] 50 mM Veronal (pH 8.7).

Results

Inactivation by Butanedione. The time course for the inactivation of creatine kinase by butanedione in 50 mM borate (pH 8.7) depends on the concentration of reagent employed (Figure 1). Under these conditions the inactivation is first order in both butanedione and creatine kinase. Semilog plots of residual activity vs. time indicate that the rate of inactivation is proportional to the concentration of butanedione and variation of the enzyme concentration from

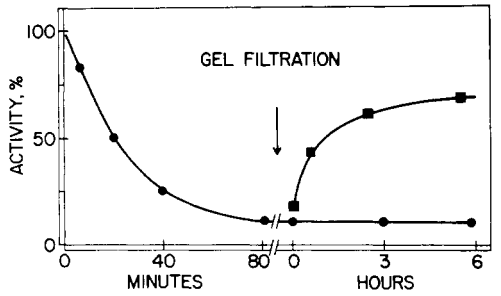

FIGURE 2: Reversibility of creatine kinase inactivation on the removal of borate. The enzyme, 7 μM, was incubated with 0.23 mM butanedione–50 mM borate (pH 8.7). After 80 min of modification, aliquots were gel filtered (arrow) on Bio-Gel P-4 in either 50 mM Tris (pH 8.7) (■), or 50 mM borate (pH 8.7) (●). The modified enzyme solutions obtained after gel filtration were incubated at 25° and periodically assayed to determine the extent of reactivation.

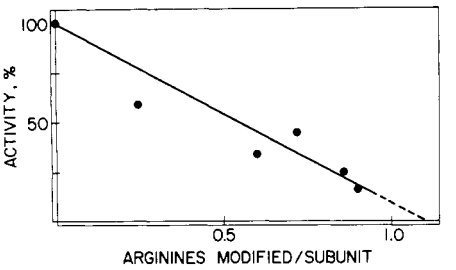

FIGURE 3: Correlation of inactivation of creatine kinase with arginine modification by butanedione. The enzyme (20 μM) was incubated with 0.14 mM butanedione–50 mM borate (pH 8.7), and aliquots were withdrawn periodically and subjected to gel filtration and subsequent analysis as described in the text. The number of arginines modified is given per subunit of enzyme.

0.6 to 15 μM (0.05–1.3 mg/ml) does not affect the rate of inactivation by 0.23 mM butanedione. A butanedione concentration of 0.23 mM reduces the activity by 50% in 19 min, corresponding to a second-order rate constant for inactivation of 156 M^{-1} min^{-1}, and these conditions were adopted for succeeding experiments.

Characteristics of the Butanedione Reaction. There is a significant effect of pH on the inactivation of creatine kinase by 0.23 mM butanedione in 50 mM borate. The rate at pH 8.7 is four times greater than that at pH 7.5 and 17 times that at pH 6.3 (Table I). This effect of pH is qualitatively similar to that observed by Riordan (1973) for carboxypeptidase A and by Lange et al. (1974) for alcohol dehydrogenase. It is likely due either to the ionization of another group on the enzyme which affects the reactivity of the essential arginyl residue or to a pH effect on the borate buffer, and would not seem to reflect the pK of the essential arginyl residue (Riordan, 1973).

There is a pronounced influence of borate concentration on the inactivation by 0.23 mM butanedione at pH 8.7 (Table II). In the absence of borate, 95% of the native activity is retained after modification for 80 min. As the borate concentration is increased, inactivation becomes more rapid until a maximal decrease in enzymatic activity occurs in the presence of 20–50 mM borate. At higher concentrations the degree of inactivation after modification for 80 min becomes progressively less, entirely in accord with the effect of borate on the butanedione reaction observed previously (Riordan, 1973).

The inactivation of creatine kinase by butanedione in borate buffer is reversible. This is demonstrated by first inactivating the enzyme to 10% of the native activity and then gel filtering to remove excess butanedione (Figure 2). If gel filtration is performed in Tris buffer, thereby removing borate as well, the activity of the modified enzyme returns progressively toward normal such that 65% of the native activity is present after 5.5-hr incubation in Tris buffer. If gel filtration is carried out in 50 mM borate, however, no reactivation is observed, and the activity remains at 10%. Reversal of modification also occurs if the enzyme is inactivated to 10% of the native activity by 0.23 mM butanedione in 50 mM borate (pH 8.7) and then diluted 50-fold into 50 mM Veronal (pH 8.7). After incubation at 25° for 24 hr, the diluted enzyme exhibits 85% of the native activity.

Chemical Consequences of Butanedione Inactivation. Amino acid analysis of creatine kinase was performed to identify the residues modified by treatment with butanedione. The ratio of lysine to histidine remains constant throughout the course of inactivation, while that of arginine to histidine decreases progressively. All other amino acid residues are unaffected. Extrapolation to complete inactivation indicates that loss of activity correlates with the modification of 1.1 arginyl residues per subunit (Figure 3).

Table III: Effect of Substrates on the Inactivation of Creatine Kinase by Butanedione.[a]

Substrate	Activity (%)
None	3
Creatine phosphate, 40 mM	12
ATP, 20 mM	55
MgATP, 20 mM	75
MgADP, 20 mM	75

[a] Creatine kinase, 3 μM, was modified by 0.23 mM butanedione in 50 mM borate (pH 8.7), 25°, for 2 hr. Aliquots were assayed and compared to the native enzyme as a control.

Protection against Butanedione Inactivation. Creatine phosphate, 40 mM, affords only slight protection against inactivation of creatine kinase by 0.23 mM butanedione (Table III). However, significant protection against inactivation is provided by 20 mM ATP and even more by 20 mM MgATP. After 2 hr, 75% of the original activity remains in the presence of MgATP compared to only 3% activity in its absence. MgADP, at the same concentration, protects against butanedione inactivation to the same degree as MgATP. Creatine, 40 mM, protects almost completely, but this is due to a reaction between its guanidino group and butanedione.

Nucleotide Binding by Butanedione-Modified Creatine Kinase. Binding of MgADP and MgATP to butanedione-modified enzyme was determined by uv difference spectroscopy (Roustan et al., 1968). Compared to the native enzyme, the modified enzyme which has lost 95% of its original activity, loses 94% of its binding capacity for MgATP and 93% of that for MgADP, as measured by the change in ΔA_{254}. Amino acid analysis of this sample reveals the loss of 1.0 arginine per subunit in the modified enzyme, and no other residue is modified. Thus, in this experiment the modification of one arginyl residue per subunit of creatine kinase leads to the loss of 95% of the native activity concomitant with the loss of approximately 94% of the nucleotide binding.

Exclusion of Cysteine Modification as a Basis for Inactivation. A number of reagents modify the single reactive

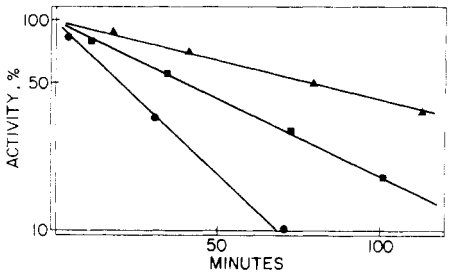

FIGURE 4: Semilog plot of changes in activity of creatine kinase, 2.5 μM, in 50 mM Veronal (pH 8.7) vs. time on reaction with 0.05 mM (▲), 0.10 mM (■), and 0.20 mM (●) phenylglyoxal. The native enzyme retains full activity over this period of time.

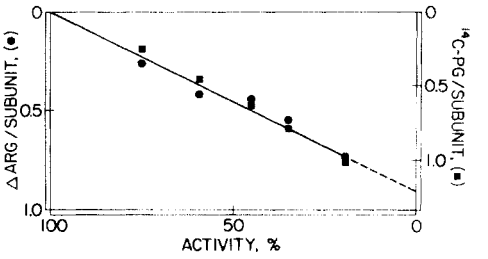

FIGURE 5: Correlation of inactivation of creatine kinase by [^{14}C]phenylglyoxal with arginine modification (●) and [^{14}C]phenylglyoxal incorporation (■). The enzyme (20 μM) was incubated with 0.20 mM [^{14}C]phenylglyoxal in 0.1 M bicarbonate (pH 8.7), and aliquots were withdrawn periodically and subjected to gel filtration and subsequent analysis as described in the text. The numbers of arginines modified and [^{14}C]phenylglyoxals incorporated are given per subunit of enzyme.

sulfhydryl group per subunit of creatine kinase with a concomitant loss of activity (for a review, see Watts, 1973). This sulfhydryl group can be titrated and blocked reversibly with 5,5′-dithiobis(2-nitrobenzoic acid) (O'Sullivan, 1971). Two sets of experiments were performed to rule out the possibility that the butanedione inactivation of creatine kinase under the above conditions might be due to a reaction with this sulfhydryl group. In one, the enzyme is inactivated by 0.23 mM butanedione in 50 mM borate (pH 8.7) and aliquots are withdrawn periodically and titrated with 5,5′-dithiobis(2-nitrobenzoic acid). The number of titratable groups remains constant throughout the course of the reaction, even when the activity decreases to less than 10% of the original value. In the other, the reactive sulfhydryl is first blocked with 5,5′-dithiobis(2-nitrobenzoic acid) and then the resultant product is treated with butanedione. Aliquots are withdrawn periodically, diluted into dithiothreitol (final concentration, 6 mM), and assayed for enzymatic activity. The activity–time profile for inactivation by butanedione under these circumstances is identical with that of the native enzyme.

Inactivation by Phenylglyoxal. The inactivation of creatine kinase by phenylglyoxal, 0.20 mM, in 50 mM Veronal (pH 8.7), proceeds with a half-life of 20 min (Figure 4). The kinetics of inactivation are apparent first-order in phenylglyoxal over the range of concentrations employed with an overall second-order rate constant for inactivation of 169 M^{-1} min^{-1}. Identical results are obtained when the modification is carried out in 100 mM bicarbonate (pH 8.7). The effects of the presence of substrates on the inactivation of creatine kinase by phenylglyoxal are very similar to those obtained with butanedione. Significant protection against inactivation is obtained with both MgADP and MgATP but not creatine phosphate.

Modification with [^{14}C] phenylglyoxal accompanied by studies of ^{14}C incorporation and amino acid analysis demonstrates the concurrent loss of activity, incorporation of 1.2 [^{14}C]phenylglyoxal molecules, and modification of 0.9 arginine residue per subunit (Figure 5). Amino acid analysis reveals no loss of lysine or any residue other than arginine. Further, the number of titratable sulfhydryl groups remains constant throughout the course of inactivation, as is the case with butanedione modification. Thus, again, loss of activity correlates with the modification of a single arginine residue per enzyme subunit and no other residues are modified.

Discussion

Arginine has been implicated as a residue essential to the function of a number of enzymes. Chemical modification of the alcohol dehydrogenases from horse and human liver, as well as from yeast, have identified arginines at the coenzyme binding sites (Lange et al., 1974), and a similar role for arginine has been demonstrated for mitochondrial malate dehydrogenase (Foster and Harrison, 1974) and lactate dehydrogenase (Yang and Schwert, 1972). Arginine modification inactivates ribonuclease A (Takahashi, 1968), inorganic pyrosphosphatase (Cooperman and Chiu, 1973), *E. coli* alkaline phosphatase (Daemen and Riordan, 1974), rabbit muscle aldolase (Lobb et al., 1975), and glutamine synthetase and carbamoyl-phosphate synthetase (Powers and Riordan, 1975). In each of these enzymes, the essential arginine is thought to interact with the negatively charged phosphate moiety of the substrate. This idea has been confirmed by X-ray crystallographic studies of staphylococcal nuclease (Arnone et al., 1971), lactate dehydrogenase (Adams et al., 1973), and horse liver alcohol dehydrogenase (Eklund et al., 1974). Lange et al. (1974) have suggested that arginyl residues play a general role as positively charged components in enzymatic recognition sites for anionic substrates. The work reported here confirms that arginyl residues function in this manner in the ATP-dependent enzyme, creatine kinase.

Butanedione is known to be highly selective for the modification of arginyl residues in proteins. This modification is augmented by borate buffer, which likely stabilizes the adduct between the guanidino group and butanedione, and the modification is reversible on the removal of excess butanedione and borate (Riordan, 1973). Creatine kinase is rapidly inactivated by butanedione in borate buffer. The modification is highly selective; inactivation can be carried out at a reagent concentration which is at least 15-fold lower than in any other study previously reported using this reagent. The almost absolute requirement for borate in the butanedione inactivation of creatine kinase (Table II) coupled with the reversal of inactivation when borate is removed completely (Figure 2) together suggest strongly that the modification of essential arginyl residues is indeed responsible for this inactivation. Amino acid analysis of modified creatine kinase substantiates the correlation between the loss of activity and the modification of 1.1 arginine residues per subunit by butanedione–borate (Figure 3).

The finding that one arginyl residue per subunit is essential for the enzymatic activation of creatine kinase was confirmed by studies utilizing a different α-dicarbonyl reagent, phenylglyoxal, which has also been shown to be highly selective for the modification of arginyl residues in proteins

(Takahashi, 1968). Phenylglyoxal rapidly inactivates creatine kinase, again in a highly selective manner. Amino acid analysis of the phenylglyoxal-modified enzyme again indicates that inactivation correlates linearly with the loss of arginine, in this case 0.9 residue per subunit (Figure 5). Thus, the conclusion is that one arginyl residue per subunit is essential for the enzymatic activity of creatine kinase.

No other residue of creatine kinase is modified by butanedione–borate or phenylglyoxal. Amino acid analysis reveals only the loss of arginine. The single, reactive sulfhydryl group per subunit (O'Sullivan, 1971) remains titratable throughout the course of inactivation by the α-dicarbonyl reagents and prior conversion of this group to a mixed disulfide with 5,5′-dithiobis(2-nitrobenzoic acid) has no effect on the rate of inactivation by butanedione–borate. Modification of this reactive sulfhydryl group, therefore, can be ruled out as the cause of inactivation by these α-dicarbonyl reagents. Moreover, when modification with [^{14}C]phenylglyoxal is monitored by both ^{14}C incorporation and amino acid analysis, virtually all of the incorporated ^{14}C can be accounted for by loss of arginine (Figure 5), assuming a 1:1 stoichiometry for the phenylglyoxal–arginine adduct. This stoichiometry differs from the 2:1 stoichiometry previously seen with this reagent (Takahashi, 1968; Daemen and Riordan, 1974; Lange et al., 1974). It may be that the first phenylglyoxal molecule inactivates the enzyme by forming a glyoxaline adduct with the guanidino group of an arginyl residue (Takahashi, 1968). However, at the phenylglyoxal concentration utilized (0.20 mM) the reaction of a second molecule to form the proposed cyclic acetal (Takahashi, 1968) proceeds only to a small degree. Steric restriction in the reaction of the second phenylglyoxal molecule by the localized environment of the specific arginine residue in creatine kinase could be an alternative explanation.

It is significant that only one of the 18 arginyl residues per subunit of creatine kinase shows a high degree of selectivity for these α-dicarbonyl reagents. Clearly, in the native enzyme this residue possesses some feature which distinguishes it from the other arginyl residues in terms of reactivity. A comparison of the rate of modification of the essential arginyl residue of creatine kinase by butanedione–borate with that of free arginine under the same conditions reveals that the arginyl residue in the enzyme reacts approximately ten times faster than the free amino acid. Since modification of only one of the 18 arginyl residues per subunit leads to complete inactivation, most or all of the 17 unmodified residues react at a rate significantly slower than free arginine. Thus, the high degree of selectivity in the butanedione–borate inactivation of creatine kinase seems to arise from a combination of both an increased reactivity of the essential arginyl residue and a decreased reactivity of most or all of the others.

This hyperactivity is also apparent when phenylglyoxal is used to modify the essential arginyl residue. In this case the reaction is approximately 15 times faster than with free arginine under the same conditions. Thus, the chemical environment of the single reactive arginyl residue per subunit enhances its reactivity toward α-dicarbonyl reagents. This high degree of selectivity in the modification of enzyme arginyl residues has been noted previously and appears to be a general phenomenon (Powers and Riordan, 1975).

The pattern of protection by the various substrates of creatine kinase is quite informative. Creatine phosphate, in which the guanidino group is chemically blocked by phosphate, protects very little against inactivation with butanedione–borate (Table III). However, creatine completely protects against inactivation. Since the dissociation constant for creatine phosphate is lower than that for creatine (8.6 mM vs. 15.6 mM) (Morrison and James, 1965), it would appear that creatine most certainly does not protect by binding to the active site of the enzyme with concomitant shielding of an arginine residue. More likely this protection is but apparent and actually due to the fact that the guanidino group of creatine reacts chemically with the butanedione, and its large excess over butanedione would effectively remove the latter from the reaction medium. On the other hand, MgATP and MgADP both offer significant protection against inactivation. Preincubation of butanedione with MgATP fails to alter the pattern of protection provided by this nucleotide, and MgATP does not react with borate. Hence, protection cannot be due to a chemical reaction between the nucleotide and the modifying agent. Protection must be due to nucleotide binding at the single active site per subunit of creatine kinase shielding an arginyl residue from modification. The lack of a uv difference spectrum on addition of MgATP or MgADP to the butanedione-modified enzyme further indicates the involvement of an arginyl residue in nucleotide binding.

Chemical modification studies have implicated several amino acid residues as being essential to the catalytic activity of creatine kinase. Thus, modification of one sulfhydryl group per subunit (Mahowald et al., 1962), one histidine per subunit (Pradel and Kassab, 1968), or one lysyl residue per subunit (Kassab et al., 1968) leads to inactivation of the enzyme. However, the role of any one of these residues in the catalytic mechanism of creatine kinase has yet to be firmly established (for a review see Watts, 1973). The work reported here not only demonstrates that creatine kinase requires one arginyl residue per subunit for activity, but defines its catalytic role. The protective effect of MgADP and MgATP against inactivation by butanedione–borate and the inability of the modified enzyme to bind MgATP indicate that the essential arginyl is located at the coenzyme binding site, providing a positive charge to interact with the negative oligophosphate moiety of the nucleotide. This postulate is strengthened by the fact that inorganic pyrophosphate, a competitive inhibitor for ATP in creatine kinase (Nihei et al., 1961), protects against inactivation by butanedione–borate to the same extent as MgATP (C. L. Borders, Jr., and J. F. Riordan, unpublished results).

These results may bear on the mechanism of ATP binding to kinases in general. Despite intensive studies on these enzymes, the amino acid residues responsible for ATP binding have remained largely unknown. From this study it may be concluded that arginine is one of the residues essential for ATP binding to creatine kinase, and preliminary studies of four other kinases—hexokinase, phosphofructokinase, phosphoglycerate kinase, and pyruvate kinase—indicate that each of these enzymes is rapidly inactivated by butanedione in borate buffer and suggest that arginyl residues may play the same role in these enzymes (C. L. Borders, Jr., and J. F. Riordan, unpublished results). Further, studies of two additional enzymes which utilize ATP other than for simple phosphate transfer, glutamine synthetase and carbamoylphosphate synthetase, show on a broader scale that arginyl residues are essential as ATP recognition sites (Powers and Riordan, 1975). Protein arginyl residues could well be involved in the ATP recognition sites of many other ATP-dependent biological activities and the chemical approaches

now available should provide a much needed means to study the importance of this residue in these systems.

References

Adams, M. J., Buehner, M., Chandrasekhar, K., Ford, G. C., Hackert, M. L., Liljas, A., Rossman, M. G., Smiley, I. E., Allison, W. S., Everse, J., Kaplan, N. O., and Taylor, S. S. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 1968.

Arnone, A., Bier, C. J., Cotton, F. A., Day, V. W., Hazen, E. E., Jr., Richardson, D. C., Richardson, J., and Yonath, A. (1971), *J. Biol. Chem. 246,* 2302.

Borders, C. L., Jr., and Riordan, J. F. (1975), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 34,* 647.

Cooperman, B. S., and Chiu, N. Y. (1973), *Biochemistry 12,* 1676.

Daemen, F. J. M., and Riordan, J. F. (1974), *Biochemistry 13,* 2865.

Davis, B. J. (1964), *Ann. N.Y. Acad. Sci. 121,* 404.

Eklund, H., Nordström, B., Zeppezauer, E., Söderlund, G., Ohlsson, I., Boiwe, T., and Bränden, C.-I. (1974), *FEBS Lett. 44,* 200.

Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82,* 70.

Foster, M., and Harrison, J. H. (1974), *Biochem. Biophys. Res. Commun. 58,* 263.

Kassab, R., Roustan, C., and Pradel, L.-A. (1968), *Biochim. Biophys. Acta 167,* 308.

Lange, L. G., III, Riordan, J. F., and Vallee, B. L. (1974), *Biochemistry 13,* 4361.

Lobb, R. R., Stokes, A. M., Hill, H. A. O., and Riordan, J. F. (1975), *FEBS Lett. 54,* 70.

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

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

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

Noda, L., Kuby, S. A., and Lardy, H. A. (1954), *J. Biol. Chem. 209,* 203.

O'Sullivan, W. J. (1971), *Int. J. Protein Res. 3,* 139.

Powers, S. G., and Riordan, J. F. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 2616.

Pradel, L.-A., and Kassab, R. (1968), *Biochim. Biophys. Acta 167,* 317.

Riley, H. A., and Gray, A. R. (1943), in Organic Synthesis, Collect. Vol. II, Blatt, A. H., Ed., New York, N.Y., Wiley, p 509.

Riordan, J. F. (1970), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 29,* 462.

Riordan, J. F. (1973) *Biochemistry 12,* 3915.

Roustan, C., Kassab, R., Pradel, L.-A., and Thoai, N. V. (1968), *Biochim. Biophys. Acta 167,* 326.

Takahashi, K. (1968), *J. Biol. Chem. 243,* 6171.

Watts, D. C. (1973), *Enzymes 3rd Ed. 8,* 383-455.

Yang, P. C., and Schwert, G. W. (1972), *Biochemistry 11,* 2218.

Yue, R. H., Palmieri, R. H., Olson, O. E., and Kuby, S. A. (1967), *Biochemistry 6,* 3204.

Effect of Ligands on the Reactivity of Essential Sulfhydryls in Brain Hexokinase. Possible Interaction between Substrate Binding Sites†

Vilas D. Redkar and Umakant W. Kenkare*

ABSTRACT: Inactivation of bovine brain mitochondrial hexokinase by 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), a sulfhydryl specific reagent, has been investigated. The study shows that the inactivation of the enzyme by DTNB proceeds by way of prior binding of the reagent to the enzyme and involves the reaction of 1 mol of DTNB with a mol of enzyme. At stoichiometric levels of DTNB, the inactivation of the enzyme is accompanied by the formation of a disulfide bond. But it is not clear whether the disulfide bond or the mixed disulfide intermediate formed prior to it causes inactivation. On the basis of considerable protection afforded by glucose against this inactivation it is tentatively concluded that the sulfhydryl residues involved in this inactivation are at the glucose binding site of the enzyme, although other possibilities are not ruled out. An analysis of the effects of various substrates and inhibitors on the kinetics of inactivation and sulfhydryl modification by DTNB has led to the proposal that the binding of substrates to the enzyme is interdependent and that glucose and glucose 6-phosphate produce slow conformational changes in the enzyme. Protective effects by ligands have been employed to calculate their dissociation constants with respect to the enzyme. The data also indicate that glucose 6-phosphate and inorganic phosphate share the same locus on the enzyme as the γ phosphate of ATP and that nucleotides ATP and ADP bind to the enzyme in the absence of Mg^{2+}.

A recent communication from our laboratory (Redkar and Kenkare, 1972) briefly described experiments bearing on the role of sulfhydryl residues in the activity of brain hexokinase. Using DTNB,[1] a thiol specific reagent, evidence was presented to indicate that one or two sulfhydryl

† From the Molecular Biology Unit, Tata Institute of Fundamental Research, Colaba, Bombay 5, India. *Received July 1, 1974.*

[1] Abbreviations used are: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid), Glc-6-P, glucose 6-phosphate; Fru-6-P, fructose 6-phosphate; TNB, thionitrobenzoate anion.