# Purification of Arginine Kinase from Lobster and a Study of Some Factors Affecting Its Reactivation*

Sandra L. Blethen† and Nathan O. Kaplan

ABSTRACT: Arginine kinase has been crystallized from the tail muscle of the American lobster (*Homarus americanus*). The preparation appeared to be homogeneous on starch gel electrophoresis at pH 8.6 and on immunodiffusion in agar gel. In the analytical ultracentrifuge, it migrated as a single symmetrical peak with a sedimentation constant of 3.25 S; in 8 M urea, a sedimentation constant of 3.1 S was observed. A molecular weight of 40,000 was calculated from sedimentation equilibrium data. The amino acid composition of lobster arginine kinase was determined; it contained five cysteine residues per molecule and no cystine. It can be reversibly inactivated by treating with 8 M urea. The reactivation of arginine kinase was studied by diluting samples from urea into buffer and measuring the increase in enzymatic activity after dilution. Reactivation is promoted by thiols and inhibited by divalent metal ions. Metal ion inhibition can be overcome by thiols or by addition of EDTA. L-Arginine or adenosine triphosphate (ATP) in concentrations sufficient to give almost complete saturation of the enzyme can also promote reactivation in the absence of thiols although they are not as effective as the thiols. Adenosine diphosphate (ADP) is less effective in promoting reactivation than either L-arginine or ATP; arginine analogs do not promote reactivation. Arginine-promoted reactivation is inhibited by metal ions.

The ability of the amino acid sequence of a polypeptide chain to determine the three-dimensional structure of its biologically active form is part of our present conception of protein biosynthesis (Epstein *et al.*, 1963). The fact that a number of enzymes have been shown to undergo reversible inactivation in the presence of substances, such as urea, guanidine, or acid which disrupt their tertiary structure provides experimental support for the role of the primary structure of a protein in determining its secondary and tertiary structures.

The studies of Anfinsen and his collaborators on the reactivation of ribonuclease (Anfinsen, 1961) are probably the best known example of reversible inactivation. These experiments involved the reduction of the disulfide bonds of the protein in urea and mercaptoethanol. Other enzymes which are reversibly inactivated by this treatment include lysozyme, taka-amylase A, pepsinogen, and alkaline phosphatase (see Epstein *et al.* (1963) for a review of the results obtained with these enzymes). Aldolase (Stellwagen and Schachman, 1962), lactic, and malic dehydrogenases (Epstein *et al.*, 1964; Chilson *et al.*, 1966) are examples of enzymes which are dissociated into inactive subunits and can reassociate and reactivate under appropriate conditions.

Although complete recovery of lost enzymatic activity should be possible if the primary structure indeed determines the active conformation, various side reactions can reduce the yield of enzyme activity obtained (Schachman, 1963). This report deals with some of the factors which influence the recovery of enzymatic activity when arginine kinase from the lobster (*Homarus americanus*) is inactivated by 8 M urea. As will be shown in this paper, arginine kinase differs from the enzymes cited as examples of reversible inactivation in that the native enzyme contains no disulfide bonds and does not appear to dissociate in 8 M urea.

Arginine kinase catalyzes the reversible transfer of a phosphoryl group from ATP[1] to L-arginine. It is widely distributed among invertebrate species where it serves a function analogous to that of creatine kinase in vertebrates. Purified preparations have been obtained from the muscle of several crustacean species: the fresh water crayfish, *Potamobius astacus* (Elodi and Szorenyi, 1956); sea crayfish, *Jasus verreauxi* (Uhr *et al.*, 1966); and the European lobster, *Homarus vulgaris* (Pradel *et al.*, 1964; Virden *et al.*, 1965). All of these enzymes require either magnesium or manganese ions for activity and are inhibited by sulfhydryl reagents, such as *p*-hydroxymercuribenzoate and iodoacetamide. The molecular weights determined for the fresh water crayfish and lobster preparations range between 36,000 and 43,000 depending on the method used in the deter-

* From the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02154. *Received September 1, 1966.* Supported in part by research grants from the National Aeronautics and Space Administration (NSG-375), American Cancer Society (P-77H, the Charles Simon memorial grant for cancer research), and the National Institutes of Health (CA-03611). Publication no. 494.

† U. S. Public Health Service Postdoctoral Fellow (1-F2-GM-30,707-01).

[1] Abbreviations used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; DPNH, reduced diphosphopyridine nucleotide.

mination (Virden *et al.*, 1966). The arginine kinase used in the present studies was crystallized from the tail muscle of the American lobster (*H. americanus*) and has a molecular weight of 40,000.

## Experimental Procedure

Arginine kinase was assayed by a modification of the method of Wiesmann and Richterich (1964). The reaction was initiated by adding enzyme solution to a cuvet with a 1-cm light path which contained the following in 0.23 M glycine (pH 8.6): $MgSO_4$ (5 mM), KCl (50 mM), ATP (2.5 mM), phosphoenolpyruvate (0.75 mM), DPNH (0.45 mM), L-arginine (15 mM), lactic dehydrogenase (12.5 μg/ml), and pyruvate kinase (10 μg/ml). The reaction was followed by measuring the decrease in absorbance at 340 mμ in a Zeiss PMQ II spectrophotometer thermostated at 30°. One unit of arginine kinase activity is defined as the amount of enzyme catalyzing the transfer of 1 μmole of phosphate from ATP to L-arginine per minute as measured during the first 2 min of the reaction. In some cases, notably when ADP was used to reactivate the enzyme, it was necessary to initiate the reaction by adding L-arginine, prewarmed to 30°, to the cuvet containing arginine kinase plus the other components of the assay system. The enzyme activity measured in this manner was the same as that obtained when the reaction was started by adding enzyme. DPNH oxidation in the absence of L-arginine was less than 0.01% of that observed with the purified enzyme when L-arginine was present.

During the course of purification, protein concentrations were determined on dialyzed samples by the method of Warburg and Christian (1941). The concentration of purified arginine kinase was calculated by using a value of 6.7 for the extinction of a 1% solution at 280 mμ (Virden *et al.*, 1965).

Starch gels were prepared in 0.017 M Tris–0.1 mM EDTA (pH 8.6) by the method of Smithies (1955). The well buffer was 0.167 M Tris–1 mM EDTA (pH 8.6). Amido Black was used to stain for protein.

Antiserum to crystalline lobster arginine kinase was prepared by the method of Plescia *et al.* (1964). Ouchterlony double-diffusion studies were performed as described by Stollar and Levine (1963). Optical rotatory dispersion studies were carried out in a Cary Model 60 spectropolarimeter equipped with a thermostated cell holder. The molecular weight of purified arginine kinase was determined by the sedimentation equilibrium method of Yphantis (1964) using 0.1 M triethylammonium hydrochloride (pH 7.0) containing 0.5% sucrose.

Amino acid analyses were performed on samples which had been exhaustively dialyzed against 0.05 M ammonium bicarbonate and lyophilized prior to hydrolysis in vacuum-sealed tubes with constant-boiling HCl according to the procedure of Spackman *et al.* (1958). Values for serine and threonine were obtained by extrapolating to zero time from the values found after hydrolysis for 24 and 48 hr. Total half-cystine was determined by measuring the cysteic acid produced after performic acid oxidation (Schram *et al.*, 1954). Cysteine was determined by titration with *p*-hydroxymercuribenzoate in 8 M urea (Boyer, 1954). The tryptophan content was calculated from the tyrosine content as determined by amino acid analysis and the tyrosine:tryptophan ratio determined in 0.1 M NaOH (Goodwin and Morton, 1946).

The peptides obtained following trypsin digestion of carboxymethylated arginine kinase were separated by two-dimensional, high-voltage paper electrophoresis at pH 6.5 and 3.5. Ninhydrin was used to locate the peptides.

Crystalline chicken heart muscle lactic dehydrogenase was prepared in this laboratory by the method of Pesce *et al.* (1964). Crystalline rabbit muscle pyruvate kinase was purchased from Boehringer, and chymotrypsin-free trypsin (B grade) was purchased from Calbiochem.

Urea from Fisher Scientific Co. was recrystallized from 95% ethanol, and all urea solutions were prepared immediately prior to use.[2] Unless otherwise noted, the procedure for studying the reactivation of arginine kinase from urea solutions was as follows. A suspension (50 μl) of crystalline lobster arginine kinase in 1.1 M ammonium sulfate was added to 0.95 ml of a solution of 8.4 M urea in 0.1 M sodium phosphate (pH 7.5)–1 mM EDTA. The solution was mixed, and after 2 min at room temperature, diluted 1:20 with 0.1 M sodium phosphate (pH 7.5). Reactivation was allowed to proceed at room temperature, and samples were removed for assay at various times. Recovery of activity was complete within 15 min after dilution, and total reactivation was measured at this time. All other chemicals used in this study were the highest grade obtainable from commercial sources and were used without further purification.

## Results

*Purification of H. americanus Arginine Kinase.* Minced lobster tail muscle (10 kg) was extracted with 30 l. of 1 mM EDTA–1 mM mercaptoethanol (pH 7.0) overnight. The extract was clarified by filtration and solid ammonium sulfate was added to bring the solution to 70% saturation. After standing overnight, the precipitate was collected by centrifugation and dissolved in 1 mM EDTA–1 mM mercaptoethanol. Solid ammonium sulfate was added to 60% saturation, and the precipitate obtained by centrifugation was dissolved in 10 mM glycine, 1 mM EDTA, and 1 mM mercaptoethanol (pH 8.0). The precipitate obtained on the addition of ammonium sulfate to 25% saturation was discarded and arginine kinase was crystallized by warming the supernatant solution to room temperature. A summary of this procedure is given in Table I.

*Physical Properties of Enzyme.* The preparation appeared to be homogeneous when subjected to starch gel

[2] When unrecrystallized urea was used to inactivate arginine kinase in the absence of EDTA, there was no reactivation in the absence of added thiol.

TABLE I: Purification of Arginine Kinase from *H. americanus* Tail Muscle.

| Fraction | Vol. (ml × $10^3$) | Act. (units/ml) | Protein (mg/ml) | Sp Act. (μmoles of phosphate transferred/min mg) |
|---|---|---|---|---|
| Crude extract | 30.0 | 448 | 14.8 | 30.5 |
| | $(NH_4)_2SO_4$ (0–70%) | | | |
| Precipitate | 1.6 | 4560 | 75.5 | 60.5 |
| Supernatant solution | 30.0 | 13 | 5.8 | 2.2 |
| | $(NH_4)_2SO_4$ (0–60%) | | | |
| Precipitate | 0.93 | 5370 | 82.9 | 65.5 |
| Supernatant solution | 1.70 | 822 | 27.2 | 30.1 |
| | $(NH_4)_2SO_4$ (0–25%) | | | |
| Precipitate | 0.39 | 2460 | 53.8 | 45.7 |
| Supernatant solution | 0.63 | 3210 | 15.2 | 212.0 |

TABLE II: Amino Acid Composition of Lobster Arginine Kinase.

| Amino Acid | Moles of Amino Acid/Mole of Protein[a] | Nearest Integral No. of Residues/Mole of Protein |
|---|---|---|
| Lysine | 26.6 | 27 |
| Histidine | 8.4 | 8 |
| Arginine | 15.7 | 16 |
| Aspartate | 37.6 | 38 |
| Threonine[b] | 16.7 | 17 |
| Serine[b] | 16.3 | 16 |
| Glutamate | 39.0 | 39 |
| Proline | 12.0 | 12 |
| Glycine | 28.1 | 28 |
| Alanine | 24.7 | 25 |
| Half-cystine[c] | 5.4 | 5 |
| Valine | 22.2 | 22 |
| Methionine | 8.2 | 8 |
| Isoleucine | 16.5 | 17 |
| Leucine | 28.2 | 28 |
| Tyrosine | 9.1 | 9 |
| Phenylalanine | 19.3 | 19 |
| Tryptophan[d] | 3.1 | 3 |
| Total | 337.1 | 333 |

[a] These values represent the average of duplicate determinations of 24- and 48-hr hydrolysates. [b] Extrapolated to zero time. [c] Determined as cysteic acid after performic acid oxidation and hydrolysis in 6 N HCl for 24 hr. [d] Calculated from the tyrosine:tryptophan ratio of 2.9.

electrophoresis at pH 8.6, and only a single precipitin line was observed on immunodiffusion in agar gel. When examined in the analytical ultracentrifuge, it migrated as a single peak with a sedimentation constant ($s_{20,w}$) of 3.25 S. In 8 M urea, it again moved as a single peak, and its sedimentation constant ($s_{20,w}$) was 3.1 S. Its molecular weight was calculated to be 40,000 from the sedimentation equilibrium data.

*Amino Acid Analyses.* The amino acid composition of lobster arginine kinase is given in Table II. Forty ninhydrin-positive spots were obtained from trypsin hydrolysates of the carboxymethylated protein as compared to the 43 different peptides which would be expected on the basis of the number of lysine and arginine residues present in the molecule.

*Inactivation of Arginine Kinase.* As can be seen from Figure 1, preincubating arginine kinase in 8 M urea decreases the initial velocity of the reaction. However, enzyme activity increases following dilution into the assay mixture although the rate of this increase is less for samples which have been in urea for longer periods of time. In order to isolate the effects of various components of the assay mixture, reactivation was studied by diluting solutions of arginine kinase from 8 M urea into buffered solutions containing the potential activator or inhibitor.

In the absence of any additions, 0.1 M sodium phosphate (pH 7.5), 0.1 M Tris-HCl (pH 7.5), 0.23 M glycine (pH 8.6), and a mixture of 0.1 M phosphate and 0.1 M glycine at pH 7.5 gave the same degree of reactivation.

*Effect of Thiol Compounds on Reactivity from Urea.* Addition of a thiol-containing compound, such as 2-mercaptoethanol to the dilution buffer, greatly increased both the total reactivation obtained (Table III) and the rate of reactivation (Figure 2). Addition of mercaptoethanol directly to an assay mixture did not increase the activity observed. Cysteine was as effective as an equimolar concentration of mercaptoethanol while dithiothreitol was effective at one-tenth the concentration (Table IV). In the presence of mercaptoethanol, the extent of reactivation was independent of protein concentrations between 7 and 70 μg/ml.

*Effect of Arginine and Other Substances on Enzyme Reactivation.* Addition of L-arginine to the dilution buffer also stimulated reactivation (Figure 2), but the ability of L-arginine to promote reactivation decreased as the length of time that the enzyme was exposed to urea increased (Table III). In the presence of mercaptoethanol, L-arginine gave no additional stimulation although addition of mercaptoethanol after the L-arginine-promoted reaction was complete further increased the reactivation obtained (Table V). If the addition of L-arginine was delayed 5 min after dilution, there was no

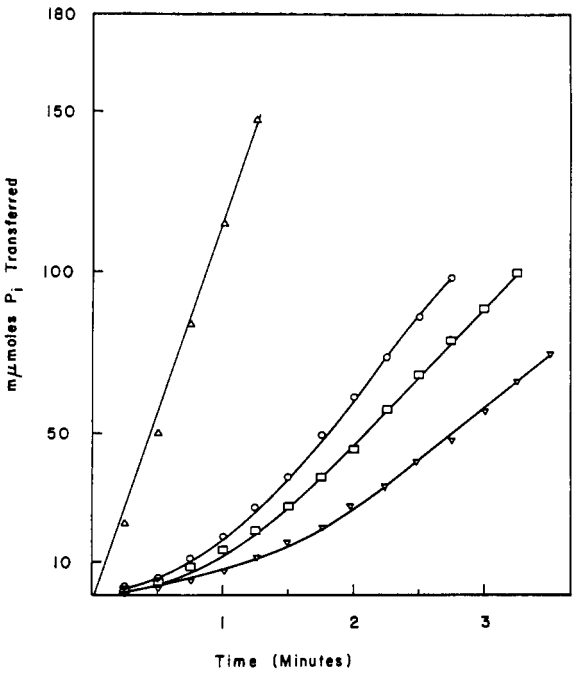



FIGURE 1: Activity of arginine kinase after treatment with 8 M urea. Stock arginine kinase (15.2 mg/ml, 212 μmoles/min mg) was diluted 1:20 with 8.4 M urea and 10-μl samples (containing 7.6 μg of protein each) were removed for assay at the times indicated. (△) Enzyme diluted 1:20 with phosphate buffer and assayed in the presence of 10 μl of 8 M urea. (○) Treated with 8 M urea for 2 min and then assayed. (□) Treated with 8 M urea for 10 min and then assayed. (▽) Treated with 8 M urea for 35 min and then assayed.

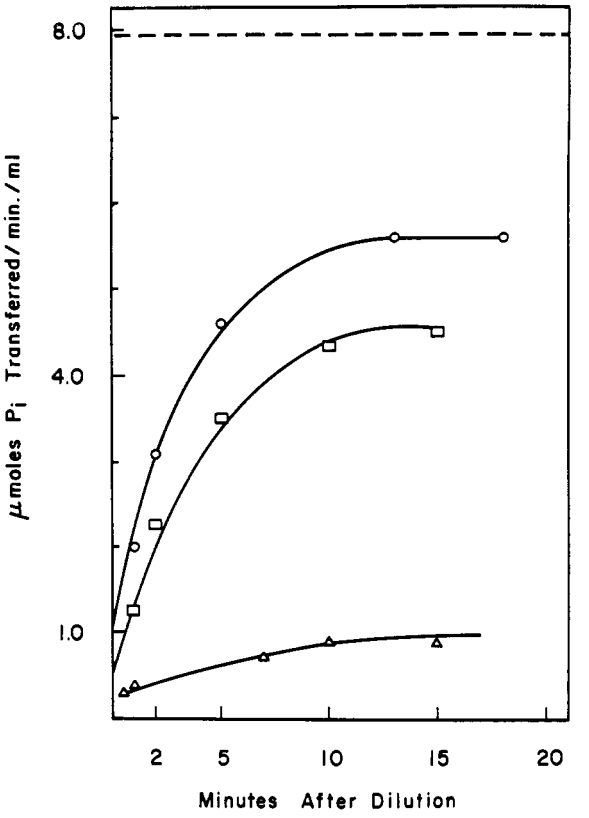



FIGURE 2: Reactivation of arginine kinase on dilution. Stock arginine kinase (15.2 mg/ml) was diluted 1:20 with 8.4 M urea; after 2 min at room temperature, a sample was removed and diluted 1:20 with 0.1 M sodium phosphate (pH 7.5) containing the additions noted. (△) No additions. (□) L-Arginine (30 mM). (○) 2-Mercaptoethanol (0.1 M). (- - -) Activity of an untreated sample.

TABLE III: Effect of Length of Time of Exposure to 8 M Urea on the Reaction of Arginine Kinase.[a]

| Time in 8 M Urea (min) | Reactivation (%) Obtained in 0.1 M $P_i$ | 0.03 M L-Arginine | 0.1 M Mercaptoethanol |
|---|---|---|---|
| 1 | 35 | 64 | 75 |
| 2 | 11 | 43 | 76 |
| 5 | 11 | 30 | 75 |
| 15 | 8 | 26 | 71 |
| 60 | 1 | 19 | 73 |

[a] See Experimental Procedure for standard reactivation conditions.

TABLE IV: Effect of Thiol Concentration on Reactivation.[a]

| Addition (mM) | Act. Recovd (%) |
|---|---|
| None | 0.9 |
| Mercaptoethanol (1) | 18.8 |
| Mercaptoethanol (10) | 42.3 |
| Mercaptoethanol (100) | 76.0 |
| L-Cysteine (100) | 70.9 |
| Dithiothreitol (10) | 74.0 |

[a] Arginine kinase was exposed to 8 M urea for 5 min and diluted 1:50 for reactivation in 0.1 M sodium phosphate (pH 7.5) containing the additions noted.

stimulation. In contrast, a delay in the addition of mercaptoethanol for up to 15 min after dilution still resulted in maximum reactivation.

The effect of substrates and of arginine analogs on reactivation is summarized in Table VI. Magnesium inhibited substrate-promoted reactivation but had no effect when mercaptoethanol was present (Table VII). Other divalent metal ions also inhibited reactivation in

TABLE V: Effect of Delayed Addition of Mercaptoethanol and L-Arginine on Total Reactivation.[a]

| Addn on Dilution (mM) | Act. Recovd (%) | Addn after Dilution (mM) | Act. Recovd (%) |
|---|---|---|---|
| None | 10.9 | None | 10.9 |
| None | 10.9 | L-Arginine (30) | 12.0 |
| None | 10.9 | Mercaptoethanol (100) | 75.0 |
| L-Arginine (30) | 40.3 | Mercaptoethanol (100) | 67.0 |

[a] Urea inactivation was carried out as described under Experimental Procedure. After 2 min in 8 M urea, samples were diluted into 0.1 M sodium phosphate buffer containing the additions noted in the first column. Five minutes after dilution, sufficient 0.3 M L-arginine was added to make the sample 30 mM with respect to arginine. Mercaptoethanol to a final concentration of 100 mM was added 15 min after dilution from 8 M urea. Final reactivation was determined 15 min after the last addition.

TABLE VI: Effect of Substrates and L-Arginine Analogs on Reactivation.[a]

| Addition (mM) | Act. Recovd (%) |
|---|---|
| None | 12.0 |
| $MgCl_2$ (5) | 3.0 |
| L-Arginine (30) | 40.5 |
| $MgCl_2$ (5) + ATP (5) | 9.0 |
| $MgCl_2$ (5) + ADP (1) | 3.0 |
| D-Arginine (30) | 17.4 |
| L-Lysine (30) | 7.9 |
| L-Ornithine (30) | 7.2 |
| Guanidoacetic acid (28) | 5.8 |

[a] See Experimental Procedure for details of the reactivation process. Addition of L-arginine, ATP Mg, or the arginine analogs directly to the assay mixture in amounts equivalent to those added in following reactivation had no effect on the enzyme activity measured.

TABLE VII: Effect of $Mg^{2+}$ on the Reactivation of Arginine Kinase by Substrates or Mercaptoethanol.[a]

| Addition (mM) | 5 mM $MgCl_2$ | Reactivation (%) | (+Mg: −Mg) |
|---|---|---|---|
| L-Arginine (30) | − | 32.3 | 2.21 |
| L-Arginine (30) | + | 14.6 | |
| ATP (5) | − | 31.4 | 2.73 |
| ATP (5) | + | 11.5 | |
| ADP (2) | − | 9.6 | 2.66 |
| ADP (2) | + | 3.6 | |
| Mercaptoethanol (10) | − | 43.0 | 1.02 |
| Mercaptoethanol (10) | + | 44.0 | |
| Mercaptoethanol (100) | − | 68 | 0.96 |
| Mercaptoethanol (100) | + | 65 | |

[a] Reactivation was carried out as described under Experimental Procedure.

TABLE VIII: Effect of Added Metal Ions on Reactivation.[a]

| Addition (mM) | Act. Recovd (%) | Inhibn in Assay (%) |
|---|---|---|
| None | 24.6 | — |
| $MgCl_2$ (5) | 1.4 | 0 |
| $MgCl_2$ (1) | 1.6 | 0 |
| $MgCl_2$ (0.1) | 3.0 | 0 |
| $MgCl_2$ (0.01) | 8.7 | 0 |
| $MnCl_2$ (5) | 1.1 | 0 |
| $CaCl_2$ (5) | 3.0 | 11.7 |
| $BaCl_2$ (5) | 2.1 | 0 |
| $BaCl_2$ (0.5) | 4.6 | 0 |
| $CuCl_2$ (5) | 0 | 0 |
| EDTA (1) | 23.5 | 0 |
| EDTA (1) + $MgCl_2$ (5) | 16.8 | 0 |

[a] These experiments were done in 0.1 M Tris-HCl, pH 7.5. The enzyme was exposed to 8 M urea for only 1 min instead of the standard 2 min. Mercaptoethanol reversed the inhibition of reactivation by metal ions except for $Cu^{2+}$ where a precipitate was formed when mercaptoethanol was added to the solution.

the absence of mercaptoethanol (Table VIII). The inhibition by divalent metal ions did not occur in the presence of EDTA, but EDTA itself did not promote reactivation.

When arginine kinase was diluted from urea into phosphate buffer in the absence of EDTA, the number of titratable sulfhydryl groups decreased. Table IX shows the effect of urea and dilution following urea treatment on the total number of titratable sulfhydryl groups in lobster arginine kinase.

*Effect of Sulfhydryl Reagents on Arginine Kinase.* Native arginine kinase was inactivated by *p*-hydroxymercuribenzoate and by iodoacetamide. L-Arginine, ATP, and ADP all protected the enzyme against these reagents, but L-arginine gave the most complete protection (Table X).

TABLE IX: Effect of Urea and Dilution on the Number of Titratable Sulfhydryl Groups in Arginine Kinase.[a]

| Treatment (M) | Titratable Sulfhydryl Groups/Mole |
|---|---|
| None | 4.5 ± 0.2 |
| Urea (8) | 4.8 ± 0.2 |
| Urea (8), diluted (1:20) in sodium phosphate (0.1) | 2.1 ± 0.2 |

[a] The values given are the average of three to five separate titrations with *p*-hydroxymercuribenzoate in 0.1 M sodium phosphate (pH 7.5). The protein concentrations used ranged from 0.17 to 0.21 mg/ml in a volume of 1.0 ml. Addition of mercaptoethanol increased the activity of preparations dialyzed against phosphate buffer in the absence of EDTA by 30%.

TABLE X: Effect of Substrates in Protecting Arginine Kinase from the Action of Thiol-Reagents.[a]

| Addition (mM) | Activity Retained (%) in Iodoacetamide | Activity Retained (%) in Hydroxymercuribenzoate |
|---|---|---|
| None | 34 | 5 |
| $MgSO_4$ (5) | 34 | 6 |
| L-Arginine (30) | 100 | 38 |
| $MgSO_4$ (5) + ATP (5) | 68 | 28 |
| $MgSO_4$ (5) + ADP (2.5) | 54 | 31 |
| L-Arginine (30) + $MgSO_4$ (5) + ATP (5) | 91 | 39 |
| L-Arginine (30) + $MgSO_4$ (5) + ADP (2.5) | 101 | 41 |

[a] *p*-Hydroxymercuribenzoate was present at a final concentration of 51.6 μM; the enzyme concentration in the experiments with *p*-hydroxymercuribenzoate was 10 μM (0.4 mg/ml). Iodoacetamide was present at a final concentration of 1 mM, and the enzyme concentration in these experiments was 12 μM (0.48 mg/ml). The buffer in both cases was 0.1 M sodium phosphate (pH 7.5). After 15 min at room temperature, the samples were diluted 1:20 and their enzymatic activity was measured in the usual way. The activity retained is expressed relative to a control which stood at room temperature for the same length of time. Under these conditions, iodoacetamide completely inactivated the enzyme in 45 min; L-arginine completely protected the enzyme from inactivation by iodoacetamide.

*Optical Rotatory Dispersion Properties of Arginine Kinase.* The optical rotatory dispersion curves of native, denatured, and reactivated lobster arginine kinase are shown in Figure 3. Because of the high blank values given by L-arginine at wavelengths below 260 mμ, the curve obtained for lobster arginine kinase reactivated in 30 mM L-arginine may not be as precise as the others shown. All of the curves obey a one-term Drude equation at wavelengths between 245 and 305 mμ; the $\lambda_c$, $b_0$, and $[m']_{233}$ values calculated from each of the curves are given in Table XI.

## Discussion

Crustacean muscle is a good source of arginine kinase since the enzyme comprises from 10 to 20% of the extractable muscle protein. The purification procedure described here is similar to the procedures used by Pradel *et al.* (1964) and Elodi and Szoreyni (1956) in purifying arginine kinase from *H. vulgaris* and *Potamobius astacus*, respectively, although the exact concentrations of ammonium sulfate required to precipitate the enzyme were different in each case.

The value of 3.25 S found here for the sedimentation constant of *H. americanus* arginine kinase is close to the value of 3.18 S reported for the enzyme from *H. vulgaris* by Virden *et al.* (1966), and that of 3.07 reported by Uhr *et al.* (1966) for the sea crayfish enzyme. However, Pradel *et al.* (1964) reported a value of 2.69 S for their preparation of lobster arginine kinase from *H. vulgaris*, and Elodi and Szorenyi (1956) found a value of 2.5 S for the arginine kinase from the fresh water crayfish. The sedimentation velocity experiments of Pradel *et al.* (1964) were conducted in 0.01 M glycine (pH 6.85), and it is probable that the low ionic strength of this buffer resulted in a reduction of the apparent sedimentation constant due to nonspecific charge interactions (Schachman, 1959). The experiments of Elodi and Szorenyi were conducted in 0.2 M glycine–1 mM KCN (pH 8.5), so that we have no ready explanation for the lower sedimentation constant of their preparation.

The sedimentation constant of 3.1 S found for lobster arginine kinase in 8 M urea is subject to uncertainties since the viscosity correction used may not be applicable in 8 M urea. However, the fact that it is close to the value observed for the native enzyme is taken as an indication that no dissociation occurs in 8 M urea, and the effects observed cannot be explained in terms of dissociation and reassociation of the protein.

The molecular weights reported for the purified preparations of arginine kinase are very similar. Virden *et al.* (1966) determined the molecular weight of *H. vulgaris* arginine kinase by a number of different methods and obtained values ranging from 36,000 to 42,000. They considered 37,000 to be the best value for their preparation while Terrossian *et al.* (1966) preferred a value of 42,000 on the basis of their sedimentation velocity experiments and the amino acid composition of their preparation. Elodi and Szorenyi (1956) calculated a molecular weight of 43,000 for their preparation using a sedimentation constant of 2.5 S and a diffusion constant of $5.6 \times 10^{-7}$ cm²/sec. The molecular weight of

TABLE XI: Rotatory Constants of Lobster Arginine Kinase.

| Solvent (M) | $\lambda_c$ (mμ) | $b_0$ | $[m']_{233}$ (deg) |
|---|---|---|---|
| Phosphate (0.1) | 237 | −103 | −3175 |
| Phosphate (0.1) + dithiothreitol (10) | 242 | −135 | −3120 |
| Urea (8.0) | 217 | 0 | −933 |
| Phosphate (0.1)[a] | 224 | −63 | −2210 |
| Phosphate (0.1), $MgCl_2$ (1)[a] | 237 | −42 | −1580 |
| Phosphate (0.1) + L-arginine (30)[a] | 247 | −56 | −1110 |
| Phosphate (0.1) + dithiothreitol (10)[a] | 226 | −81 | −2225 |

[a] Dissolved in 8 M urea for 2 min before dilution in the solvent given.

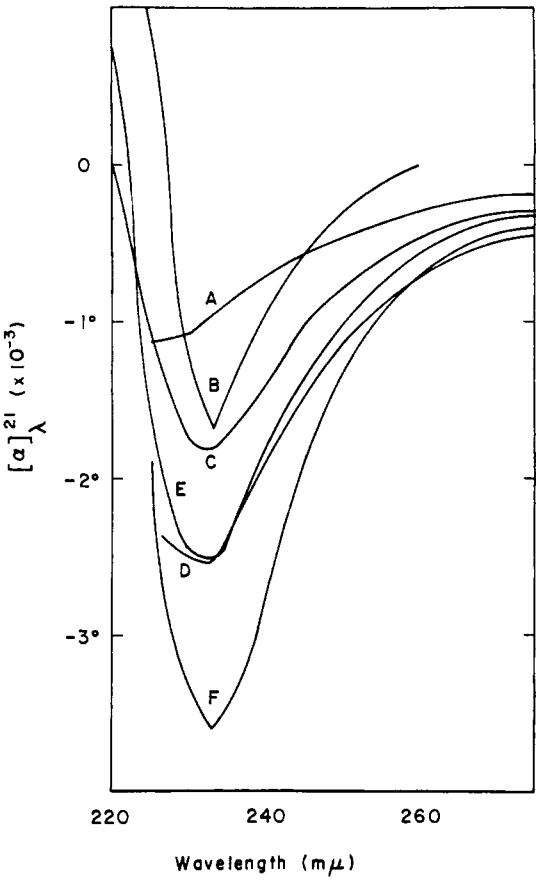


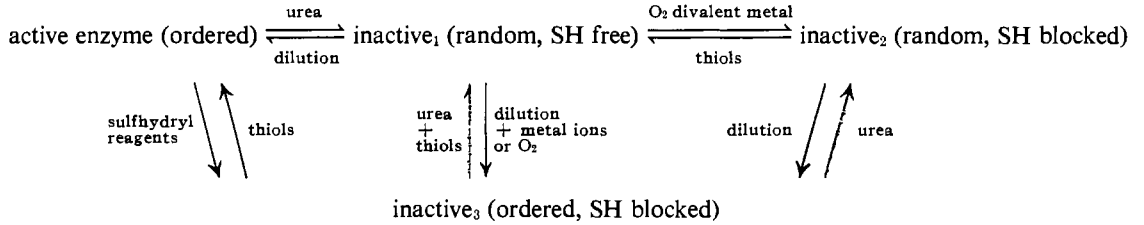

FIGURE 3: Optical rotatory dispersion curves of arginine kinase. (curve A) Arginine kinase in 8 M urea (0.13 mg/ml). (curve B) Arginine kinase treated with 8 M urea for 2 min and diluted 1:10 with 30 mM L-arginine (0.23 mg/ml). (curve C) Arginine kinase treated with 8 M urea for 2 min and diluted 1:10 with 1 mM $MgCl_2$ (0.22 mg/ml). (curve D) Arginine kinase treated with 8 M urea for 2 min and diluted 1:10 into 10 mM dithiothreitol (0.22 mg/ml). (curve E) Arginine kinase treated with 8 M urea for 2 min and diluted 1:10 with phosphate buffer (0.23 mg/ml). (curve F) Native arginine kinase (0.13 mg/ml). All curves were obtained at temperatures between 21 and 22°.

40,000 reported for the *H. americanus* enzyme is similar to these values.

The total number of half-cystine residues per mole was found to be 5.4 while there were 4.8 titratable cysteines. Since the preparation used to determine the cysteine content could be activated by the addition of mercaptoethanol, the amount of cysteine may have been underestimated. Virden and Watts (1966) also noted a decrease in the number of titratable sulfhydryl groups on storage of their preparation, but in this case there was no loss of enzymatic activity. In any case, the difference between the number of half-cystine and cysteine residues per mole is considerably less than 2, and this fact indicates that there are no disulfide bonds present in *H. americanus* arginine kinase. When considered with the fact that the sedimentation constant of lobster arginine kinase does not change appreciably in 8 M urea and the fact that the number of peptides obtained on tryptic digestion of arginine kinase agrees closely with the number which would be expected from the number of lysine and arginine residues present, this lack of disulfide bonds indicates that the protein molecule is composed of a single polypeptide chain.

The importance of a sulfhydryl group in the arginine binding site of *H. vulgaris* arginine kinase has been demonstrated (Pradel *et al.*, 1965; Virden and Watts, 1966). Earlier studies (Pradel *et al.*, 1964) have also implicated sulfhydryl groups in the nucleotide binding region, and the experiments of Virden and Watts (1966) suggest that free sulfhydryl groups may be important in maintaining the active conformation of the enzyme. The data presented in Table X indicate the importance of a free sulfhydryl group in the activity of *H. americanus* arginine kinase and suggest that in those instances where only a thiol-containing compound was able to reverse inactivation, return of the protein to its active form was prevented by the blocking of a formerly free sulfhydryl group. The decrease in the number of titratable sulfhydryl groups per mole of enzyme following dilution from urea supports this hypothesis as do the optical rotatory dispersion studies. These studies indicate that arginine kinase molecules diluted from urea into phosphate buffer recover as much of their helical structure as do molecules diluted into thiol-containing solutions (see curves D and E, Figure 3). It appears that the protein molecules are capable of re-forming a significant part of their native structure on dilution from urea independently, and that part of the role of substances in promoting the recovery of activity is in protecting the sulfhydryl groups of the protein. Thiols can also promote reactivation by reversing the blocking of sulfhydryl groups. The fact that a delay in the addition of mercap-

SCHEME I




toethanol does not decrease its effectiveness in promoting reactivation while a delay in the addition of L-arginine does is also in accord with this hypothesis. The ability of mercaptoethanol to increase the reactivation obtained over that given by L-arginine can be explained by the inability of arginine to reverse the blocking of sulfhydryl groups.

The ability of L-arginine to promote reactivation is consistant with its ability to protect the native enzyme from the action of thiol reagents. However, ADP and ATP protect the enzyme from sulfhydryl reagents to a lesser extent, and yet ATP is about as effective as L-arginine in promoting reactivation while ADP is ineffective in increasing the reactivation obtained after dilution. Thus, while the maintenance of free sulfhydryl groups is important for reactivation, protection of sulfhydryl groups alone cannot explain all of the substrate effects noted.

It also appears that molecules diluted from urea into L-arginine do not recover as much helical structure as molecules diluted into phosphate buffer, but this effect may be the result of subtracting the large blank value given by arginine alone from the curves obtained with the protein and L-arginine. Of the amino acids and guanidinium compounds tested, L-arginine was the only one capable of promoting reactivation. This is probably a reflection of the specificity of its binding site on the enzyme as none of the other compounds was able to act as a substrate for arginine kinase.

The inhibition of reactivation by magnesium and manganese ions is especially interesting in view of the fact that the presence of one of them is required for enzymatic activity. Magnetic resonance studies (W. J. O'Sullivan, 1966, personal communication) indicate that *H. americanus* arginine kinase is similar to rabbit muscle creatine kinase in binding the metal ions as a metal–nucleotide complex. There is also an indication that the protein is capable of binding manganese ion alone, but whether this binding is a property of the native or denatured protein is still questionable. The inhibition of reactivation observed in the presence of these ions may be due to nonspecific metal ion binding, and this binding may prevent full recovery of helical structure as indicated by the optical rotatory dispersion data. Since EDTA alone did not promote reactivation when recrystallized urea was used, it appears that the presence of trace metal contaminants did not cause the loss in activity found under the standard conditions.

The nature of the reactions leading to the loss of free sulfhydryl groups is not as yet clear. The formation of cyanate in urea solutions at neutral or alkaline pH is well known (Stark *et al.*, 1960), but the use of freshly prepared solutions and short exposure times should eliminate carbamylation of free sulfhydryl groups as a cause of lost activity. Another possibility is the formation of disulfide bonds by the sulfhydryl groups of the unfolded protein. Since there are no disulfide bonds in the native enzyme, this type of disulfide bond formation is opposite in effect from the "correct" re-formation of disulfide bonds necessary for the reactivation of reduced ribonuclease.

Scheme I summarizes the data presented in this paper with respect to the reactivation of arginine kinase from *H. americanus*. In this diagram, the dotted lines indicate reactions which have not been studied, but which probably could occur under proper circumstances. One role of L-arginine and ATP would be to prevent the conversion of inactive$_1$ into inactive$_3$, but as indicated above, these substrates may have an additional function in promoting reactivation which is at present unknown.

## References

Anfinsen, C. B. (1961), *J. Polymer Sci. 49*, 31.

Boyer, P. D. (1954), *J. Am. Chem. Soc. 76*, 4331.

Chilson, O. P., Kitto, G. B., Pudles, J., and Kaplan, N. O. (1966), *J. Biol. Chem. 241*, 2431.

Elodi, P., and Szorenyi, E. (1956), *Acta Physiol. Acad. Sci. Hung. 9*, 367.

Epstein, C. J., Carter, M. M., and Goldberger, R. F. (1964), *Biochim. Biophys. Acta 92*, 391.

Epstein, C. J., Goldberger, R. F., and Anfinsen, C. B. (1963), *Cold Spring Harbor Symp. Quant. Biol. 28*, 439.

Goodwin, T. W., and Morton, R. A. (1946), *Biochem. J. 40*, 628.

Pesce, A., McKay, R., Stolzenbach, F., Cahn, R., and Kaplan, N. O. (1964), *J. Biol. Chem. 239*, 1753.

Plescia, O. J., Braun, W., and Palczuk, N. C. (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 279.

Pradel, L. A., Kassab, R., Regnouf, F., and Thoai, N. V. (1964), *Biochim. Biophys. Acta 89*, 255.

Pradel, L. A., Kassab, R., Terrossian, E., and Thoai, N. V. (1965), *Compt. Rend. 260*, 3212.

Schachman, H. K. (1959), Ultracentrifugation in Biochemistry, New York, N. Y., Academic, pp 225–228.

Schachman, H. K. (1963), *Cold Spring Habor Symp. Quant. Biol. 28*, 409.
Schram, E., Moore, S., and Bigwood, E. J. (1954), *Biochem. J. 57*, 33.
Smithies, O. (1955), *Biochem. J. 61*, 629.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Stark, G. R., Stein, W. H., and Moore, S. (1960), *J. Biol. Chem. 235*, 3177.
Stellwagen, E., and Schachman, H. K. (1962), *Biochemistry 1*, 1056.
Stollar, D., and Levine, L. (1963), *Methods Enzymol. 6*, 848.
Terrossian, E., Kassab, R., Pradel, L. A., and Thoai, N. V. (1966), *Biochim. Biophys. Acta 122*, 462.
Uhr, M. L., Marcus, F., and Morrison, J. F. (1966), *J. Biol. Chem. 241*, 5428.
Virden, R., and Watts, D. C. (1966), *Biochem. J. 99*, 162.
Virden, R., Watts, D. C., and Baldwin, E. (1965), *Biochem. J. 94*, 536.
Virden, R., Watts, D. C., Watts, R. L., Grammock, D. B., and Raper, J. H. (1966), *Biochem. J. 99*, 155.
Warburg, O., and Christian, W. (1941), *Biochem. Z. 310*, 384.
Wiesmann, U., and Richterich, R. (1964), *Helv. Physiol. Pharmacol. Acta 22*, 1.
Yphantis, D. A. (1964), *Biochemistry 3*, 297.

# The Activation of Papain and Ficin by Phosphorothioate*

Hava Neumann, Meir Shinitzky, and Roberts A. Smith

ABSTRACT: Phosphorothioate (PSH) was found to activate papain and ficin in an equimolar ratio. One molecule of PSH is reversibly incorporated into the protein molecule with the formation of one free sulfhydryl group, which is directly related to the enzymic activity of papain and ficin, as was demonstrated by the reaction of iodoacetic acid with the above proteins. The equilibrium constant, $K$, for the activation of papain was evaluated from (a) the change in activity of papain upon the addition of small quantities of PSH, and (b) the simultaneous decrease in fluorescence intensity. The value obtained by the two methods was found to be $6.0 \times 10^6$ l./mole at 23°. The value of the enthalpy change accompanying the reaction, $\Delta H$, was derived from activation curves at various temperatures. $\Delta H = -9.8$ kcal/mole. The decay of fluorescence of papain after a rapid addition of PSH is of the order of seconds, and therefore the kinetics of the interaction between papain and the activator could be evaluated from fluorescence decay curves. The kinetic studies showed that the reaction between papain and PSH is a second-order reaction with a rate constant $k_1 = 3.2 \times 10^3$ sec$^{-1}$ mole$^{-1}$ l. This is consistent with the observation that 1 mole of PSH reacts with 1 mole of papain. The rate constant of the decomposition of the papain–PS complex, $k_{-1}$, as calculated from the equation $K = (k_1/k_{-1})$, is equal to $5.3 \times 10^{-4}$ sec$^{-1}$, which corresponds to a half-life of 21 min for the isolated papain–PS complex. The half-life of the isolated papain–PS complex, obtained after gel filtration, was found to be 28 min, which is in good agreement with the value obtained from the fluorescence studies. The equilibrium constant, $K$, for the reaction between PSH and ficin was found to be $K = 6.5 \times 10^6$ l./mole at 23°.

It is now accepted that papain and ficin possess a single reactive sulfhydryl group, which is essential for activity, and which probably participates in the catalytic action of the enzyme (Smith and Kimmel, 1960; Smith, 1958b). Furthermore, it is generally agreed that papain and ficin are activated by reduction (Smith, 1958a; Finkle and Smith, 1958). The study presented here was prompted by the fact (Neumann, 1965) that papain and ficin are activated by phosphorothioate in a 1:1 molar ratio. Phosphorothioate was shown in previous papers to be a reducing agent (Neumann *et al.*, 1965) and to cleave disulfide bonds in small model molecules (Neumann, 1965) and in proteins (Neumann *et al.*, 1964). In this work we studied the mode of activation of papain and ficin by phosphorothioate.

## Experimental Section

### *Materials*

*Papain and Ficin.* Twice-crystallized-papain and ficin prepared by the Worthington Biochemical Corp. were used. Every batch used was checked for activity in the absence of activator. Only batches that showed

* From the Department of Biophysics, The Weizmann Institute of Science, Rehovoth, Israel (H. N. and M. S.), and the Department of Chemistry, University of California, Los Angeles, California (R. A. S.). *Received January 18, 1967.*