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The Purification and Properties of Rabbit-Muscle Myokinase

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Previous methods for purifying myokinase (adenylate kinase) have made use of the procedure of Colowick & Kalckar (1943), which has been improved by Noda & Kuby (1955, 1956), Bendall (1954) and Bowen & Kerwin (1955). A different method, to be described here, yields a preparation which appears to be very similar in its physical properties to the crystalline enzyme of Noda & Kuby (1956). A study has also been made of the properties of the sulphydryl groups of the enzyme and of its heat stability.

Preliminary reports of this work have been given elsewhere (Callaghan, 1956, 1957).

EXPERIMENTAL

Materials and methods

Adenosine triphosphate (ATP). This was obtained as the disodium salt from the Sigma Chemical Co., St Louis, Mo., U.S.A.

Adenosine diphosphate (ADP). This was prepared by the dephosphorylation of ATP by means of the potato adenylyl pyrophosphatase of Lee & Eiler (1951), and the barium salt was prepared according to Le Page (1951). This was stored at -15° . Chromatographic analysis indicated that 94.5% of the phosphate was present in the form of ADP, 1% as adenosine 5'-monophosphate (AMP) and 4.5% as inorganic phosphate.

Adenosine 5'-monophosphate. AMP was obtained from Roche Products Ltd., Welwyn Garden City, England. Solutions of AMP were brought to pH 7 before use.

Sodium creatine phosphate hexahydrate. This was a gift from the Sigma Chemical Co.

L-Cysteine hydrochloride. The preparation (British Drug Houses, London) was dissolved in water and carefully neutralized to pH 7.

Sodium p-chloromercuribenzoate. This was a gift from Dr G. Weber.

Methylmercury nitrate. This was prepared from methylmercuric iodide by treatment with silver nitrate in ethanol (Johns, Peterson & Hixon, 1930). The product was twice recrystallized from water, and had a melting point of $59-60^{\circ}$. Methylmercury iodide was prepared from mercury and methyl iodide by the photochemical method of Maynard (1932), and, after two crystallizations from alcohol, had a melting point of 144° . The combined methods gave a yield of 70% of methylmercury nitrate.

Haemoglobin and cytochrome c. These were gifts of Dr F. W. J. Teale.

Hexokinase and glucose 6-phosphate dehydrogenase. A mixture of these enzymes ('coupling enzyme') was obtained from yeast by the method of Oliver (1955). The freeze-dried preparation was stored in the refrigerator.

Crystalline bovine-plasma albumin. This was obtained from Armour Laboratories, Lindsey St., London, E.C. 1.

Spectrophotometer. The Unicam spectrophotometer model SP. 500 was employed for the determination of optical density. At 340 m μ , glass cuvettes (1 cm.) were used and quartz cuvettes (1 cm.) were used to measure optical density at 260 and 280 m μ .

In assaying enzyme activity, the temperature of the cuvettes was controlled by a water-jacketed cell compartment made according to Campbell & Simpson (1953).

Routine assay of myokinase. The enzyme was assayed by the method of Oliver (1955). This method gave duplicate results which agreed within $\pm 5\%$ of each other, and was invaluable for the rapid assay of the enzyme in any large series of experiments. The method depends on the reactions:

$2\text{ADP} \rightarrow \text{ATP} + \text{AMP}$; $\text{ATP} + \text{glucose} \rightarrow \text{ADP} + \text{glucose 6-phosphate}$; $\text{glucose 6-phosphate} + \text{oxidized triphosphopyridine nucleotide (TPN)} \rightarrow \text{6-phosphogluconic acid} + \text{reduced TPN}$.

The rate of reduction of TPN was measured by observing the increase in optical density at $340\text{ m}\mu$ with time. The following constituents were added to a test cuvette: 2.8 ml. of 0.075 M -sodium diethyl barbiturate-HCl buffer, pH 8.6, containing 0.1 M -KCl, 0.01 M - MgCl_2 and TPN (3 mg./20 ml.); 0.2 ml. of 0.02 M -ADP solution; 0.2 ml. of 0.5 M -glucose; 0.2 ml. of 'coupling enzyme' soln. (20 mg./ml. in barbitone buffer). On addition of the coupling enzyme, a small 'blank' rate of extinction increase was observed. Suitably diluted myokinase in cysteine solution (0.1 ml.) was added, and the rate measured at 30 sec. intervals. All readings were measured against a blank cuvette containing 0.2 ml. of coupling enzyme soln. and 2.8 ml. of buffer. The steady rate of reaction was obtained from a plot of optical density against time. At 30° , a rate of increase of extinction at $340\text{ m}\mu$ of $0.1/\text{min.}$ (corrected for blank activity) under the above conditions was arbitrarily chosen as the unit of enzyme activity. It was found that, at this velocity, the coupling enzymes were in sufficient excess to ensure that the myokinase reaction was the rate-determining factor.

Chromatographic assay. A more precise measurement of enzyme activity was performed as follows: the enzyme was activated with cysteine, as described below, and added to a buffered solution containing Mg^{2+} ions and the requisite concentration of adenosine phosphates at 30° . After incubation for 1 min. , and at subsequent times, samples of this mixture were treated with 0.1 vol. of 30% (w/v) trichloroacetic acid and cooled to 0° .

These samples, which could be safely stored at -15° , were then analysed chromatographically to separate ATP, ADP, AMP and inorganic phosphorus by the method of Krebs & Hems (1953); the individual nucleotides on chromatograms were wet-ashed, and the phosphorus content was determined by the method of Berenblum & Chain (1938).

The following digestion mixture was found to ash the paper with great rapidity: 1.25 g. of ammonium molybdate (AnalaR) in 10 ml. of water was added to 50 ml. of $\text{H}_2\text{SO}_4\text{-HClO}_4$ ($3:2$, v/v). On standing overnight, a crystalline precipitate settled. A portion (0.5 ml.) of the supernatant was used to ash each specimen.

Protein estimation. The protein concentration in enzyme solutions was determined both spectrophotometrically and by measurements of dry weight.

For the determination of protein concentration in the presence of nucleotides, the approximate formula of Kalkar (1947) was applied: $1.45.E(280\text{ m}\mu) - 0.74.E(260\text{ m}\mu) = \text{mg. of protein/ml. (in a 1 cm. cuvette)}$. Purified myokinase was dialysed exhaustively against water, and spectrophotometric and dry-weight determinations showed that the enzyme gave an optical density, $E_{1\text{ cm.}}^{1\%}(280\text{ m}\mu) = 11.1$.

Electrophoresis. The isoelectric point of the purified enzyme was determined by paper electrophoresis in 0.03 M -phosphate buffers, with an EEL apparatus.

Myokinase was also analysed in the zone electrophoresis apparatus of Flodin & Porath (1954). A buffer of 0.0115 M -borate and 0.0155 M - NaH_2PO_4 , pH 8.4 ($I 0.05$) was used. The column of the apparatus was packed with acetylated cellulose, prepared according to Campbell & Stone (1956).

Purification of myokinase. All operations were carried out in the cold. Fresh rabbit muscle was chilled in cracked ice and minced twice. The mince was extracted with $1.5\text{--}2\text{ vol.}$ of 0.05 M -KCl for 20 min. , and the centrifuged extract was dialysed for 3 hr. against cold running water and then against 20 times its volume of distilled water at 4° for 6 hr. The fractionation of the dialysed extract followed the method of Askonas (1951). The extract was adjusted to pH 6.5 with NaHCO_3 and cooled in a stainless-steel vessel to 0° with constant stirring. Propanol was added in a fine jet at a rate compatible with the maintenance of low temperature. After 20% (v/v) has been added, the temperature was lowered to -5° , and the propanol added to a final concentration of 40% (v/v). After equilibration for 20 min. the protein precipitate was centrifuged at -5° in pre-cooled containers; the supernatant was discarded and the precipitate immediately suspended and broken up in ice-cold 0.033 M -phosphate buffer, pH 7.4, and dialysed against the same buffer for 3 hr. A flocculent precipitate appeared which contained the enzyme. This was collected and the supernatant discarded. The precipitate was suspended in cold water (0.25 vol./vol. of original extract), dissolved at pH 2 by the dropwise addition of 2 N -HCl and treated with 0.04 vol. of 5 M -NaCl, and readjusted to pH 6.5 with 2 N -NaOH, when a dense precipitate reappeared. The clear supernatant was a stable solution of the enzyme. The precipitate could again be extracted with HCl, as described above, for further enzyme. The combined solutions were fractionated on a column of calcium phosphate gel (Swingle & Tiselius, 1951), Whatman cellulose powder being used instead of the conventional inert packing material (Hyflo Supercel). A mixture of 4 parts of cellulose powder to 1 part of gel (dry wt.) was used. A column $15\text{ cm.} \times 6\text{ cm.}^2$ was adequate for the protein derived from 500 g. of muscle. The solution was driven into the column with O_2 at a pressure of 20 lb./in.^2 After the protein on the column had been washed with 0.2 M -NaCl to remove the bulk of nucleotides present, a single peak absorbing light at $280\text{ m}\mu$ (see Fig. 1) was eluted with 0.025 M -phosphate buffer, pH 7, in 0.2 M -NaCl, and 14 ml. fractions were collected in polythene tubes. The enzyme peak was followed by a trail of inactive protein. Fractions which displayed enzymic activity were combined and fractionated with $(\text{NH}_4)_2\text{SO}_4$, the fraction which precipitated between 0.65 and 0.85 saturation being centrifuged down at $25\,000\text{ g}$ and dissolved in 0.2 M -NaCl. Successive

Fig. 1. Protein content and enzyme activity of fractions eluted from a column of calcium phosphate gel (see text). ○, Optical density at $260\text{ m}\mu$; ●, optical density at $280\text{ m}\mu$; △, protein concentration; ■, enzyme activity.

Table 1. *Details of a representative preparation of myokinase (see text)*

Treatment	Total units	Total protein (mg.)	Volume (ml.)
Extract from mince	1 550 000	8450	545
Dialysed extract	1 470 000	8250	610
Solution of enzyme in NaCl, obtained after propanol fractionation and subsequent treatment with HCl and NaOH	435 000	95	125
Further solution of enzyme in NaCl after re-extraction of precipitate with HCl	232 000	42.5	150
Combined fractions effluent from $\text{Ca}_3(\text{PO}_4)_2$ column	620 000	67.5	68
0.65–0.85 saturated $(\text{NH}_4)_2\text{SO}_4$. Enzyme dissolved in 0.2M-NaCl	605 000	55	10

Fig. 2. Protein content and enzyme activity of 6 ml. fractions eluted from zone-electrophoresis apparatus after electrophoresis for 7 hr. 20 min. at 140 v D.C. at 5° in borate-phosphate buffer (0.0115M- $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ –0.0155M- NaH_2PO_4 ; pH 8.4; I 0.05). ●, Optical density at 280 mμ; ■, enzyme activity.

Fig. 3. Schlieren diagrams obtained at various times during centrifuging in a Spinco analytical ultracentrifuge of 1% solution of myokinase in 0.2M-phosphate buffer, pH 6.5. Speed, 50 730 rev./min. (186 000 g). (a) 32 min.; (b) 48 min.; (c) 64 min.; (d) 240 min.

preparations of myokinase were equally pure after this step. The activity and purity data for a representative preparation are given in Table 1.

Storage and dialysis of the enzyme. Solutions of myokinase in 0.2M-NaCl or neutral phosphate buffer are stable for several weeks at 2°, if kept in polythene tubes. For reasons

which are dealt with below, glass vessels should not be used. The enzyme is also inactivated on dialysing in cellophan sacs. Bendall (1954) reported that myokinase could be dialysed, without loss of activity, in collodion tubes coated with haemoglobin. Collodion tubes prepared by the method of Adair (1925) as described by Gutfreund (1952) may be coated with cytochrome *c* or haemoglobin by contact of the inner surface of the tube with a 1–2% solution of either protein for 48 hr. Such coated tubes may be used for the dialysis of myokinase, with small loss of activity. Nine out of ten such tubes were found to be satisfactorily coated for the safe dialysis of myokinase.

RESULTS

Properties of myokinase

The myokinase preparation described here gave both biuret and nitroprusside reactions, indicating that it is a sulphhydryl-containing protein. The enzyme is stable over the range pH 6–9. Its stability in acid is described more fully below.

Zone electrophoresis (Fig. 2) indicated that the preparation contained a single protein component. From electrophoresis on paper it was estimated that the enzyme is isoelectric at pH 4.3; this figure differs from the value pH 6.05 obtained by Noda & Kuby (1956) by an unspecified method, but as myokinase is partially inactivated during paper electrophoresis and tends to adhere to the paper, the value pH 4.3 should be regarded as a tentative one.

Myokinase appears to be free from bound nucleotides, the ultraviolet-light absorption having a ratio $E_{280 \text{ m}\mu}/E_{260 \text{ m}\mu} = 1.28$. The optimum pH value for enzyme activity has not been determined precisely, but the highest activity was observed at pH 7.5. Over the range pH 7–8 the activity does not fall below 65% of the maximum.

Myokinase shows a single component in the ultracentrifuge (Fig. 3). The sedimentation coefficient of a 1% solution in water (S_{20}) was 1.7×10^{-13} . Noda & Kuby (1956) report a value $S_{25} = 2.3 \times 10^{-13}$ for the crystalline enzyme on 0.15M-KCl. Corrected for electrolyte, viscosity and temperature, this corresponds to a value S_{20}

(water) = 1.8×10^{-13} . These same authors have assigned a molecular weight of 21 000 to the enzyme, based on sedimentation and diffusion measurements. The writer has found a unit molecular weight of approx. 10 800/sulphydryl group, from titration with methylmercury nitrate (see below); thus a molecular weight of 21 600 may be postulated.

Titration of the sulphydryl groups of myokinase. Sulphydryl groups were titrated with methylmercury nitrate by essentially the same method as that of Edelhoch, Katchalski, Maybury, Hughes & Edsall (1953). A solution of 5M-guanidine hydrochloride was adjusted to pH 10 with solid anhydrous Na_2CO_3 and filtered. Ethylenediaminetetra-acetate was added to 0.01M-concentration of this substance. To 2 ml. of the cold guanidine reagent 0.5–1 ml. of myokinase solution (5 mg./ml.) and 1 drop of fresh 10% sodium nitroprusside were added, and the purple colour was titrated to a colourless end-point. The titration was carried out rapidly, near 0°, with approx. 10^{-3} M-methylmercury nitrate which had been standardized against freshly diluted thioglycollic acid. The thioglycollic acid (British Drug Houses Ltd., 97% pure) was itself standardized by acidimetry against NaOH solution with thymolphthalein indicator.

Three portions of myokinase solution, each containing 5.4 mg. of enzyme, gave 0.500, 0.500 and 0.458 μmole of sulphydryl. For a molecular weight of 21 000 (Noda & Kuby, 1956) these quantities were equivalent to 1.94 and 1.78 SH groups/molecule. Conversely, a unit molecular weight of $11\,000 \pm 500$ /sulphydryl group could be estimated.

A further sample of enzyme, which had been treated with the guanidine reagent containing 10^{-3} M-*p*-chloromercuribenzoate, failed to give the nitroprusside reaction and no titration was possible.

Samples (0.5 ml.) of the dialysed muscle extract (see Table 1), which contained 14 mg. of protein/ml., contained 0.72–0.74 μmole of SH. Since the enzyme content of this extract (by assay) was not more than 3–4% of the total protein, a 35–44-fold excess of sulphydryl groups over those possessed by myokinase was present.

Inhibition by p-chloromercuribenzoate. Equimolar concentrations of enzyme and *p*-chloromercuribenzoate showed approx. 40–50% inhibition of enzyme activity. This inhibition, which is reversed by addition of cysteine, will be the subject of a further paper.

Ultracentrifuge studies. The possibility was entertained that oxidation of SH groups in myokinase might lead to disulphide linkages being formed between different molecules. This would lead to the appearance of polymers of higher molecular weight.

Schlieren diagrams, obtained in the ultracentrifuge, of the fully active enzyme and the enzyme

after total oxidation (but not irreversible inactivation) by dialysis for 48 hr. in cellophan gave S_{20} values (water) of 1.7×10^{-13} in both cases. No additional components were detected for the oxidized enzyme.

Velocity of forward reaction. The speed of the forward reaction $2\text{ADP} \rightarrow \text{ATP} + \text{AMP}$ decays rapidly with time (Fig. 4). By drawing tangents to the progress curve, by the mirror method (Joos, 1934),

Fig. 4. Decay of velocity of reaction $2\text{ADP} \rightarrow \text{ATP} + \text{AMP}$ at 30° with time (see text). At zero time, the reaction mixture consisted of 0.075M-2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, pH 7.5, 0.0045M-ADP, 0.002M- MgCl_2 , 0.006M-cysteine hydrochloride and 1 μg of myokinase, in a total volume of 4.5 ml. Portions were withdrawn at various times and treated with 0.1 vol. of 30% trichloroacetic acid. The line *MM* indicates the position of the mirror (see text).

Fig. 5. Lineweaver-Burk plot for determination of K_m at 30° (see text). Myokinase (0.5 μg .) was used and the reaction stopped after 1 min. by treatment with 0.1 vol. of 30% trichloroacetic acid. At zero time the reaction mixtures consisted of 0.075M-tris buffer, pH 7.5, 0.002M- MgCl_2 , 0.006M-cysteine hydrochloride. Total volume, 1.6 ml. Intercept at $-1/K_m$ indicates a K_m value of 1.6×10^{-3} M.

it can be shown that a tangent drawn to the curve at the point of 10% dismutation has a slope which is 56% of that of the initial tangent. The velocity falls to 22% when 15% of the ADP is dismuted. It is essential therefore to measure the rate of myokinase activity at very short time intervals before an appreciable fraction of the ADP is dismuted.

The velocity v determined graphically from the initial tangent in Fig. 4 was obtained with a substrate concentration of $4.5 \times 10^{-3} \text{ M}$. The maximum velocity (V) was derived from this by the equation $V = v(K_m + [S])/[S]$. The K_m value (Fig. 5) is $1.6 \times 10^{-3} \text{ M}$ [this value for K_m , obtained by the graphical method of Lineweaver & Burk (1934), is similar to the figure $1.3 \times 10^{-3} \text{ M}$ reported by Siekevitz & Potter (1953) for the mitochondrial myokinase of rat liver]. Experimentally, $v = 1.9 \mu\text{-moles of ADP}/\mu\text{g. of protein/min. at pH 7.5 and } 30^\circ$. Therefore $V = 2.5 \times 10^5$ moles of ADP/ $10^5 \text{ g. of protein/min.}$ With an enzyme molecular weight of 21 000 the turnover number is therefore 52 500, or for a molecular weight of 21 600 the turnover number is 54 000.

Effect of pH on the reaction equilibrium. Fig. 6 shows the values of the equilibrium constant $K = [\text{ATP}][\text{AMP}]/[\text{ADP}]^2$ obtained at various conditions of pH. Each value is an average figure obtained by approaching equilibrium from both sides of the reaction. It will be seen that at low pH values, where the ionization of ADP is suppressed, the enzymic dismutation of ADP is facilitated as compared with the reverse reaction.

Properties of the sulphydryl groups of myokinase and the stability of the enzyme. It was originally

reported by Colowick & Kalckar (1943) that myokinase contained SH groups, the oxidation of which resulted in the disappearance of enzymic activity. Full restoration of activity was obtained on adding reduced glutathione or cysteine. Our partially purified myokinase was entirely inactivated after dialysis for 48 hr. in cellophan tubing. Activity was not restored by addition of a concentrate of the dialysate, but the enzyme was almost entirely reactivated on addition of cysteine. Although partially purified solutions of myokinase were stable in glass vessels, the purest preparations were

Fig. 6. Effect of pH on equilibrium of myokinase reaction (see text) after incubation for 4 hr. at 30° . At zero time, the reaction mixture (4 ml.) consisted of 0.075 M-2-amino-2-hydroxymethylpropane-1:3-diol buffer, 0.015 M-ADP or 0.0075 M-ATP + 0.0075 M-AMP, 0.01 M-MgCl₂, 0.006 M-cysteine hydrochloride and 1 $\mu\text{g.}$ of myokinase. The reaction was stopped by adding 0.1 vol. of 30% (w/v) trichloroacetic acid.

Table 2. *Stability of myokinase solutions in various types of containers (see text)*

The solutions were stored at 5° and neutral pH.

Preparation	Concn. (mg./ml.)	Treatment		Activity recovered (%)	Activity recovered on adding cysteine (1 mg./ $\mu\text{g.}$ of protein)
		Nature	Duration		
Purified 25-fold	0.63	Stored in glass	3 weeks	100	100
	0.22	Stored in glass	3 weeks	95	95
	0.63	Stored in glass	4 weeks	95	95
	0.63	Dialysed in cellophan	48 hr.	0	95
	0.22	Dialysed in cellophan	48 hr.	0	90
	0.69	Stored in glass	1 week	0	—
Purified 60-fold	0.69	Stored in glass	96 hr.	0	40
	0.69	Dialysed in cellophan	48 hr.	0	90
	2.7	Dialysed in cellophan	48 hr.	0	95
	2.7	Dialysed in collodion bag	48 hr.	10	80
	1.28	Dialysed in collodion bag coated with cytochrome c	72 hr.	100	100
	2.7	Dialysed in collodion bag coated with cytochrome c	48 hr.	50	90
	10.5	Dialysed in collodion bag coated with haemoglobin	48 hr.	85	100
	2.7	Dialysed in collodion bag coated with haemoglobin	48 hr.	60	90
	0.69	Stored in polythene tube	8 weeks	100	100
	0.69	Stored in Lusteroid tube	8 weeks	80	90

inactivated by contact with both glass and cellophan. In these cases, partial restoration of activity was obtained by adding cysteine, but prolonged exposure, especially to glass, resulted in irreversible inactivation (i.e. not modified by cysteine) and the appearance of a protein precipitate with fibrous strands, which imparted a flow birefringence to the solution.

Table 2 summarizes the effect of glass and cellophan on myokinase solutions, as well as the efficacy of storage in polythene and Lusteroid vessels. For kinetic experiments, polythene tubes are suitable, and the enzyme should be first activated by incubating the requisite enzyme sample at room temperature with neutral cysteine solution (1 mg. of cysteine/ μ g. of myokinase/ml.).

Acid and heat stability. Colowick & Kalekar (1943) established that the myokinase activity of muscle extracts was largely stable to heating at 90° for 1–2 min. in 0.05N-HCl, and that a preparation approx. four times purified retained 79% of its activity after 10 min. at 100° in 0.1N-HCl. These authors treated the reneutralized enzyme solution with reduced glutathione before assay, from which it would appear that there was inactivation after the heat-acid treatment, although no figures were given.

Table 3 shows the degree of inactivation of highly purified myokinase after heating in acid. Heating resulted in a loss of activity, but activity was recovered on subsequent addition of cysteine. If excess of cysteine was present during the heating not only was greater activity detected, but further treatment with cysteine had little effect. It appears that the first effect of heating is to cause oxidation of SH groups; the oxidized enzyme then undergoes irreversible inactivation or denaturation. The stabilizing effect of accompanying proteins on myokinase has been reported by Bowen & Kerwin

(1956). On the basis of the above experiment, the present author believes that the protective action of such proteins is due to their ability to reduce the sulphhydryl groups of myokinase at the expense of their own. The results given in Table 4 show the protective action of three sulphhydryl-containing proteins on myokinase. The results are also consistent with the view that sulphhydryl-containing proteins in the crude muscle extracts, from which myokinase is purified, are responsible for the greater stability of the enzyme in such solutions, since the purer preparations of enzyme are more prone to irreversible denaturation over long periods (see Table 2). Titration of sulphhydryl groups in crude extracts (see above) indicated that there is a 40-fold excess of sulphhydryl groups over those belonging to myokinase.

Effect of salts and other proteins on heat stability. Both myokinase and actomyosin were precipitated by heating in the absence of ions, the former being almost entirely irreversibly inactivated (Tables 3 and 4). Variable amounts of myokinase were recovered if HCl was present, however, and over 90%

Table 3. *Effect of heat and acid on myokinase activity, and the protective action of cysteine*

The enzyme (82 μ g., highly purified) was heated in a total volume of 2 ml. at 100° for 10 min. After cooling in ice-water, and neutralizing to pH 7, portions were removed for spectrophotometric assay. All heating was carried out in polythene tubes.

Additions	Activity recovered (%)	Activity recovered (%) after treatment with cysteine
None	0	15
HCl (0.1N)	20	40
HCl (0.1N) + 2 mg. of cysteine	70	70
HCl (0.1N) + 2 mg. of cysteine	85	90

Table 4. *Protective effect of proteins on myokinase solutions*

The myokinase, highly purified, was heated in a total volume of 2 ml. at 90° for 10 min., cooled in ice-water and neutralized, pH 7. All heating was carried out in polythene tubes. The actomyosin preparation (Szent-Gyorgyi, 1951) was free of myokinase activity. Control experiments also showed that simple addition of the proteins to myokinase solutions did not interfere with the recovery of enzyme activity. All figures of recovery represent the average value of duplicate experiments.

Amount of enzyme (μ g.)	Other proteins added	0.1N-HCl	0.16M-NaCl	Recovery (%)	Recovery (%) after treatment with cysteine
82	None	—	+	57.5	72.5
82	None	—	—	0	12.5
82	None	+	+	0	15
82	None	+	—	0	12.5
82	300 μ g. of actomyosin	—	+	60	92.5
82	300 μ g. of actomyosin	—	—	10	15
82	300 μ g. of actomyosin	+	+	50	65
82	300 μ g. of actomyosin	+	—	62.5	80
82	300 μ g. of haemoglobin	+	—	60	80
82	300 μ g. of preheated actomyosin	+	—	25	42.5
90	300 μ g. of albumin (single expt.)	+	+	25	50

of the activity was recovered if NaCl was present. The failure of actomyosin and of pre-heated myosin to preserve myokinase activity under salt-free conditions was probably due to precipitation of these added proteins, or to a coprecipitation with myokinase, since it is known that the latter is strongly adsorbed by myosin.

Effect of heating in glass tubes. A comparison of the effects of heating the enzyme for 10 min. in polythene or glass tubes showed that similar recoveries of enzyme were obtained. Since glass has been shown to cause oxidation of the pure enzyme, whereas polythene does not (Table 2), it is inferred that heat itself causes oxidation and irreversible inactivation.

DISCUSSION

From the foregoing description of the properties of myokinase it is apparent that the sulphhydryl groups of the enzyme determine the state of activity. Two processes are involved in the denaturation of the enzyme. The first stage involves a reversible oxidation of sulphhydryl groups. In the second stage, the oxidized enzyme is irreversibly inactivated, resulting in aggregation and insolubility. Myokinase in its active form is largely stable to heating in acid. The oxidized enzyme is more easily denatured. The absence of ions appears to render the enzyme less stable, but the primary condition for denaturation appears to be oxidation. The action of glass as a denaturing agent has not been further investigated. It is possible that traces of metals in solution are responsible for oxidation, and that glass may yield such trace metals.

The protective action of added proteins appears to be due, not to physical forces such as adsorption of the enzyme by van de Waals's or ionic forces, but to their possession of a specific chemical group, the sulphhydryl, which keeps in the reduced state the sulphhydryl groups of myokinase. This explanation for the protective effect of added proteins on myokinase may well be found to apply to other cases of protection of enzymes by inert proteins.

Since myokinase appears to possess two sulphhydryl groups/molecule and does not polymerize on oxidation, the oxidation may involve the formation of a disulphide link in each molecule.

SUMMARY

1. The preparation of myokinase, homogeneous by electrophoretic and ultracentrifugal analysis, and of constant specific activity is described.

2. Pure myokinase is very readily oxidized by exposure to glass or cellophan; such treatment predisposes the enzyme to denaturation. The reactivation of the oxidized enzyme with cysteine shows that the enzyme contains highly reactive sulphhydryl groups.

3. Protection of the sulphhydryl groups with cysteine, or other sulphhydryl-containing proteins, endows the enzyme with considerable stability to heating and acid conditions.

4. Titration of the sulphhydryl groups of myokinase indicated that the enzyme possesses two groups/molecule. A molecular weight of 21 600 is postulated.

5. Ultracentrifuge studies indicated that myokinase does not polymerize on oxidation.

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