STUDIES ON A PHOSPHOPROTEIN PHOSPHATASE DERIVED FROM BEEF SPLEEN*

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SUMMARY

1. A modification of the purification procedure of SUNDARARAJAN AND SARMA for beef spleen phosphoprotein phosphatase is described which yields a highly active soluble and stable enzyme preparation.

2. The enzyme cleaves phosphate from nucleotides and from aromatic phosphate esters as well as from phosphoproteins. Other substrates include phosphorylenol pyruvate, phosphoamide, 3-phosphoglyceric acid and fructose-1,6-diphosphate.

3. Enzyme activity is considerably enhanced by reducing agents, ascorbic acid and ferrous ion being the most effective activators. No other metal was found to stimulate the enzyme. Enzyme activity is inhibited by low concentrations of molybdate, 1,10-*o*-phenanthroline, α,α'-dipyridyl and 8-hydroxyquinoline.

4. The enzyme is inactivated by short exposure to reducing agents in the absence of substrates. Ferrous ion protects against this inactivation. A hypothesis to explain these observations is presented.

INTRODUCTION

A study of a phosphoprotein phosphatase derived from beef spleen[2,3] was undertaken in the hope that this enzyme would prove to be a useful tool for the detection and study of various phosphorylated proteins which might be formed as intermediates during oxidative phosphorylation and other phosphate transfer reactions. Since purified preparations of phosphoprotein phosphatase have been shown by SINGER AND FRUTON[4] to cleave phosphoamides and phenyl phosphate as well as phosphoprotein, it was thought desirable to reexamine the substrate specificity of a highly purified preparation particularly in respect to ATP and to characterize its properties before employing the enzyme as a tool in such studies. While the present work was in progress, HOFMAN[5] reported that a protein phosphatase from beef spleen having about one-fifth the specific activity of that of SINGER AND FRUTON cleaved ATP and acetyl phosphate at a rapid rate. More recently the further work of SUNDARARAJAN AND SARMA[6] has confirmed these observations. It is the purpose of this paper to describe the properties of phosphoprotein phosphatase purified from beef spleen by a modification of the procedure of SUNDARARAJAN AND SARMA[2,3], which resulted in

* A preliminary report was presented before the American Society of Biological Chemists[1].

a soluble and stable preparation of high specific activity, and to present additional evidence for the identity of the protein responsible for the cleavage of casein, ATP and several other phosphate esters.

MATERIALS AND METHODS

Substrates

Casein (Hammersten) was obtained from Mann Research Laboratories, Inc., New York. 10 g of casein were dissolved in 85 ml of water with the addition of 16 ml of 0.4 *N* sodium hydroxide and the solution was dialyzed overnight against two changes of 20 volumes of 0.05 *M* acetate buffer of pH 5.8. Phosvitin was a gift of Dr. M. RABINOWITZ. *o*-phosphoserine peptides were kindly supplied by Dr. O. MELLANDER. *β*-casein phosphopeptone was donated by Dr. R. PETERSON. Glucose-6-phosphate (Ba), glucose-1-phosphate (diK), the nucleoside mono-, di-, and triphosphates, DPN and *p*-nitrophenyl phosphate were products of Sigma Chemical Company, St. Louis, Missouri. DL-o-phosphothreonine, phosphorylenol pyruvate (Ag, Ba), creatine phosphate, bis(*p*-nitrophenyl) phosphate, and *o*-carboxyphenyl phosphate were obtained from the California Foundation for Biochemical Research, Los Angeles, California. *β*-naphthol phosphate, phenyl phosphate and phosphoamide were generously supplied by Dr. J. CHANLEY. Ribose-5-phosphate, acetyl phosphate and fructose-1,6-diphosphate were products of Schwarz Laboratories, Mt. Vernon, New York. D, L, and DL-o-phosphoserine were kindly given by Dr. M. NEMER. Fructose-1-phosphate and 3-phosphoglyceric acid were products of Boehringer and Soehne, Mannheim, Germany. Cellulose DEAE Type 20 ion exchange agent[7] was a product of the Brown Company, Berlin, New Hampshire.

Analytical procedures

Inorganic phosphate was determined according to LOHMANN AND JENDRASSIK[8], unless otherwise specified. Protein was measured by the quantitative biuret procedure of ROBINSON AND HOGDEN[9] and spectrophotometrically according to WARBURG AND CHRISTIAN[10].

Phosphoprotein phosphatase activity was measured in a test system containing 8 *μ*moles of casein bound phosphate, 100 *μ*moles of acetate buffer of pH 5.8, 5 *μ*moles of neutralized ascorbic acid and enzyme in a total volume of 1 ml. After incubation for 15 or 30 min at 37°, the reaction was stopped by the addition of 1 ml of ice cold 20 % trichloroacetic acid and the amount of inorganic phosphate liberated was determined on an aliquot of the supernatant solution after centrifugation. Release of inorganic phosphate from casein was proportional to the concentration of purified enzyme and linear with time under the conditions employed.

Enzyme activity towards ATP was measured in a similar test system in which 5 *μ*moles of ATP replaced casein as substrate.

A unit of activity is defined as the liberation of 1 *μ*mole of inorganic phosphate/min. Specific activity is expressed as units/mg of enzyme protein as measured by the biuret reaction.

Acid soluble nitrogenous material released when casein was the substrate was measured with Nessler reagent according to KOCH AND MCMEEKIN[11] after digestion with sulfuric acid and hydrogen peroxide.

Enzyme preparation

Modification of the purification procedure of SUNDARARAJAN AND SARMA[2,3] to include removal of nucleic acid by protamine, followed by further purification on a cellulose DEAE column, yielded a soluble enzyme preparation. All operations were carried out at 4° unless otherwise specified.

Step 1. Preparation of crude extract: Six beef spleens collected in ice were freed of capsular material and ground in a meat grinder. Six batches of 400 g of minced tissue were homogenized with 1 l of a 0.5 *M* sodium chloride–0.2 *M* acetate solution of pH 5.0 in a large Waring blendor with a capacity of 7.5 l and overhead drive. The homogenate was centrifuged immediately at 1,000 × *g* for 15 min and the supernatant solution was filtered through several layers of gauze (4,600 ml).

Step 2. Ammonium sulfate fractionation: 22.6 g of solid ammonium sulfate/100 ml of crude extract were added and after stirring for 30 min the mixture was centrifuged at 16,000 × *g* for 20 min. The precipitate was discarded and 26 g of solid ammonium sulfate/100 ml of supernatant solution were added. After 30 min the mixture was centrifuged as before and the precipitate was taken up in a minimum volume of water (about 300 ml). Dialysis with stirring against 8 l of glass distilled water (5 to 6 changes) was continued (about 48 h) until the dialysate was free of sulfate ion. The precipitate formed during dialysis contained the major portion of the enzyme and was separated from the supernatant solution by centrifugation. The precipitate was extracted twice with 0.5 *M* sodium chloride–0.2 *M* acetate solution of pH 5.0, spinning after each extraction at 100,000 × *g* for 45 min in a Spinco centrifuge. The ratio of the u.v. extinction of the combined extracts (178 ml) at 280 mμ and 260 mμ was 0.65–0.72 indicating the presence of about 11 to 14% nucleic acid.

Step 3. Heat and protamine sulfate treatment: The reddish solution was divided into 15-ml aliquots and heated for 5 min in test tubes in a water bath maintained at 70°. The solutions were then quickly chilled in an ice bath and centrifuged to remove denatured protein. The clear amber supernatant solution contained over 90 % of the total activity of the unheated enzyme, and about 25% of the protein. However, the ratio of u.v. extinction at 280 mμ and 260 mμ did not change significantly. Attempts at further purification of this preparation on a DEAE cellulose column were not reproducible because of the presence of the nucleic acid and several peaks of activity emerged on fractional elution. Consequently, the enzyme solution was treated with protamine sulfate. To 164 ml of the heated enzyme, 15 ml of a 2% protamine sulfate solution were added at room temperature and after 10 min the mixture was centrifuged at 8,000 × *g* for 15 min. The enzyme was precipitated from the supernatant solution, which retained all the activity, by the addition of 56 g of solid ammonium sulfate/100 ml. After centrifugation the enzyme was taken up in about 8 ml of water and dialyzed overnight against 3 l of 0.005 *M* sodium chloride–0.002 *M* acetate solution of pH 6.0. A precipitate which formed contained no activity and was discarded. The clear yellow-brown supernatant solution (17.8 ml) had a ratio of u.v. extinction at 280 mμ and 260 mμ of 1.35.

Step 4. Fractionation on a cellulose DEAE column: The enzyme solution was applied to a DEAE cellulose anion exchange column (5 × 3.5 cm), which had been equilibrated with 0.005 *M* sodium chloride–0.002 *M* acetate solution of pH 6.0. 10-ml fractions were collected. In contrast to the nucleoprotein preparation before protamine treatment, the enzyme did not adhere to the cellulose and was eluted in tubes 4,

5 and 6 upon washing with the equilibrating buffer solution. The ratio of u.v. extinction at 280 mμ and 260 mμ of the purified enzyme at this stage was 1.45.

RESULTS

Specificity of the purified enzyme

A summary of a typical purification of the enzyme is presented in Table I. Activity towards ATP, a common substrate in phosphate transfer reactions, as well as towards the phosphoprotein casein, was followed throughout the purification procedure. It can be seen that the ratio of ATPase to casein phosphatase activity remained constant throughout the latter steps of the preparation, suggesting that these two activities reside in the same protein. At a concentration of 2 μmoles/ml casein was completely dephosphorylated by 25 μg of the purified spleen enzyme in 1 h without the release of significant amounts of acid soluble nitrogenous material (less than 0.05 mole of nitrogen/mole of phosphate released). When ATP was the substrate the two terminal phosphates were liberated.

A survey of the substrate specificity of the purified enzyme preparation is summarized in Table II. In confirmation of previous reports[4-6] the enzyme attacked most rapidly the two terminal phosphates of ATP and other nucleotides, as well as aromatic phosphate esters. Inorganic pyrophosphate and phosphoproteins were hydrolyzed at about 1/6 the rate of ATP. Phosphorylenol pyruvate was found to be an excellent substrate which was split about twice as fast as casein. The rapid cleavage of acetyl phosphate has been reported previously[5]. Phosphoamide was hydrolyzed at about 0.5 the rate of casein. Others[4] have observed a somewhat more rapid hydrolysis of this substrate, but experimental conditions were not strictly comparable. In particular, creatine phosphate was cleaved only sluggishly with the enzyme preparation described in the present paper. Some aliphatic phosphate esters, such as fructose-1,6-diphosphate, adenosine-5'-phosphate and 3-phosphoglyceric acid, were cleaved more rapidly than creatine phosphate. The observed hydrolysis of 3-phosphoglyceric acid at 0.1 the rate of casein is contrary to previous reports and will be discussed later. Among the substrates attacked comparatively slowly are phosphoserine and phosphothreonine, which were hydrolyzed at about 1 % the rate of casein. Phosphoserine peptides were hydrolyzed somewhat faster, the rate apparently increasing with the chain lengths of the peptides.

Activation of enzyme by reducing agents

In agreement with the observations of previous investigators[2,4], it was found that enzyme activity was considerably enhanced by the presence of reducing agents. As various compounds have been employed, it was of interest to compare their stimulation of the casein phosphatase activity. It can be seen in Fig. 1 that ascorbic acid and thioglycollic acid were the best activators, glutathione was barely active, while α-thioglycerol, cysteine and 2-mercaptoethanol were intermediate in their effectiveness. Experiments with another enzyme preparation showed a somewhat lower activation by cysteine and 2-mercaptoethanol, ascorbic acid yielding maximal results and glutathione being ineffective. With ATP as substrate ascorbic acid was also most effective, while thioglycollic acid was only about half as active at the optimal concen-

TABLE I

PURIFICATION OF PHOSPHOPROTEIN PHOSPHATASE

Step	*Volume ml*	*Protein g*	*Activity (Casein) Units*	*Specific activity (Casein) Units/mg protein*	*Activity (ATP) Units*	*Specific activity (ATP) Units/mg protein*	*ATP/Casein*
1	4,600	165	3,550	0.022	26,700	0.16	7.3
2	178	2.3	1,000	0.44	6,200	2.7	6.1
3	17.8	0.178	600	3.4	3,570	20.0	5.9
4	33	0.096	600	6.3	3,620	38.0	6.0

TABLE II

SPECIFICITY OF PHOSPHOPROTEIN PHOSPHATASE

Test system: 5 μmoles of substrate, 100 μmoles of acetate buffer of pH 5.8, 5 μmoles of neutralized ascorbic acid and 10 μg of enzyme (specific activity with casein = 6.0) in a final volume of 1 ml incubated at 37° for 15 or 30 min. Meaning of the number in brackets: (1) Phosphate release from pyrophosphate was divided by 2, since two moles of inorganic phosphate are released for each mole of substrate cleaved. (2) Measured by the appearance of *p*-nitrophenol[12] and of inorganic phosphate. (3) Disappearance of DPN was measured spectrophotometrically with alcohol dehydrogenase. (4) Measured by a modification of the LOWRY-LOPEZ procedure[13,14] with color development at ice bath temperature to reduce the rate of non-enzymic hydrolysis[15]. (5) Measured as the change in creatine phosphate concentration (acid-labile phosphate) after removal of inorganic phosphate by $CaCl_2$ precipitation[16,17]. (6) Measured as the change in acetyl phosphate concentration by the hydroxamic acid procedure of LIPMANN AND TUTTLE[18].

Substrate	*µg phosphate liberated*		*Substrate*	*µg phosphate liberated*	
	15 min	*30 min*		*15 min*	*30 min*
Phosphoproteins			Pyrophosphates		
Casein	26	48	ATP	78**	
Phosvitin		39	CTP*	52**	
			GTP*	19.5**	
Phosphopeptides			ADP	78**	
Ser–P·gly		0.4	UDP*	78**	
Gly·ser–P		0.8			
Gly·ser–P·gly		2.4	Inorganic pyrophosphate	25 (1)	
β-casein phosphopeptone	4				
			Phosphodiesters		
Phosphomonoesters			Bis(*p*-nitrophenyl) phosphate		1.2 (2)
Glucose-6-phosphate		2.1	DPN		0 (3)
Glucose-1-phosphate		0			
Fructose-6-phosphate		1.3	Phosphoamides		
Fructose-1-phosphate		1.8	Phosphoamide		23 (4)
Fructose-1,6-diphosphate		8.6	Creatine phosphate		1.5 (5)
Ribose-5-phosphate		2.9			
Ribulose-1,5-diphosphate	9.3		Acyl phosphate		
Adenosine-5′-phosphate		11.0	Acetyl phosphate	26 (6)	
α- or β-glycerol phosphate		0.5			
3-phosphoglyceric acid		5.4	Enol phosphate		
Phosphoserine (D, L, DL)		0.5	Phosphorylenol phosphate	50	
Phosphothreonine (DL)		0.6			
β-naphthol phosphate	69**				
p-nitrophenyl phosphate	34**(2)				
o-carboxyphenyl phosphate	50**				
Phenyl phosphate	41**				

* 3.3 mg of substrate in 1 ml.
** 5 μg of enzyme used.

tration of 10^{-2} *M*. In view of these data, ascorbic acid ($5 \cdot 10^{-3}$ *M*) was selected for routine assay procedure.

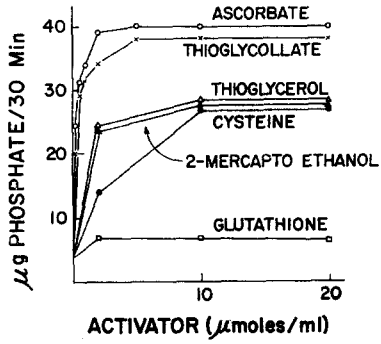

Fig. 1. Activation of casein phosphatase by reducing agents. Each test tube contained in a final volume of 1 ml, 100 μmoles of acetate buffer of pH 5.8, 8 μmoles of casein phosphate, 8 μg of enzyme (specific activity with casein = 6.0) and reducing agent as indicated. After 30 min incubation at 37°, the reactions were stopped by the addition of 1 ml of cold 20% trichloroacetic acid and the samples were analyzed for inorganic phosphate.

Effect of metals

The reducing agents could be completely replaced by ferrous ion, which was as effective as ascorbic acid, giving maximal stimulation at 10^{-3} *M* with casein and at $4 \cdot 10^{-4}$ *M* with ATP as substrate. The difference in concentration of metal ion required for optimal activity with these two substrates may be simply a reflection of the binding capacity of casein for iron[19], thus reducing the effective concentration available to the enzyme. Higher concentrations of ferrous ion showed less stimulation and actually inhibited enzyme activity at a concentration of $5 \cdot 10^{-3}$ *M*.

Metals commonly associated with phosphate transfer reactions: calcium, magnesium and manganese, did not stimulate enzyme activity with either casein or ATP when tested in the absence of reducing agents and became inhibitory at about 10^{-2} *M*. Cu^{++}, Co^{++}, Ni^{++}, Zn^{++} or Fe^{++} did not stimulate either and in fact caused about 50% inhibition at about 10^{-3} *M*.

Effect of inhibitors

In line with this suggestive evidence of a metal participant, various chelating agents were found to inhibit enzyme activity. Table III summarizes the effect of various inhibitors with ATP and casein as substrates. Molybdate was the most effective agent, giving 50% inhibition at about $4 \cdot 10^{-6}$ *M* and complete inhibition at 10^{-4} *M*. 1,10-*o*-phenanthroline; α,α'-dipyridyl and 8-hydroxyquinoline, which are particularly effective in sequestering iron[20], were all effective inhibitors. With the exception of fluoride the extent of inhibition by each compound was the same with both substrates.

p-chloromercuribenzoate, even at high concentration (10^{-3} *M*), inhibited ATPase only by 40%. Casein phosphatase activity was even less susceptible and only if the enzyme was preincubated for 15 min with the inhibitor before the addition of the substrate, suggesting that casein may bind this inhibitor. Also, high concentrations of iodoacetate (10^{-2} *M*) gave only 30% inhibition of the casein phosphatase activity with or without preincubation.

Phosphate, a reaction product, gave 40 % inhibition of casein phosphatase activity at 10^{-2} *M*. Azide, cyanide and ethylenediamine tetraacetate were without effect below 10^{-2} *M* on the ascorbic acid activated enzyme with either ATP or casein as substrate.

TABLE III

EFFECT OF INHIBITORS ON CASEIN PHOSPHATASE AND ON ATPASE ACTIVITY

Each test tube contained in a final volume of 1 ml, 100 μmoles of acetate buffer of pH 5.8, 5 μmoles of neutralized ascorbic acid, either 5 μmoles of ATP or 8 μmoles of casein phosphate, inhibitor as indicated and 10 μg of enzyme (specific activity with casein = 5.0). After incubation at 37° for 15 min, the reactions were stopped by the addition of 1 ml of 20 % trichloroacetic acid and the samples were analyzed for inorganic phosphate. Data are expressed as % inhibition of phosphate release.

Inhibitor *M*	*Phenanthroline*		*α,α'-dipyridyl*		*8-hydroxyquinolin*		*Molybdate*		*Fluoride*	
	*Casein**	*ATP*	*Casein**	*ATP*	*Casein**	*ATP*	*Casein*	*ATP*	*Casein*	*ATP*
10^{-7}	—	—	—	—	—	—	0	0	—	—
10^{-6}	0	0	0	0	0	0	25	30	—	—
10^{-5}	55	58	0	0	0	0	76	72	—	—
$4 \cdot 10^{-5}$	68	71	54	47	0	10	—	—	—	—
10^{-4}	68	71	75	62	25	30	97	92	—	—
$4 \cdot 10^{-4}$	69	71	79	74	40	45	—	—	—	—
10^{-3}	70	71	84	73	49	42	100	100	18	58
$4 \cdot 10^{-3}$	—	—	—	—	—	—	—	—	52	84
10^{-2}	—	—	—	—	—	—	—	—	63	89

* Casein was dialyzed first against 100 volumes of 0.002 *M* ethylenediamine tetraacetate and then against 100 volumes of 0.05 *M* acetate buffer of pH 5.8 before use.

Inactivation of enzyme activity by reducing agents

It was observed that temperature equilibration of enzyme with buffer and reducing agent for variable time intervals before starting the reaction by the addition of substrate resulted in variable activity. Investigation of this phenomenon revealed that the activating agents ascorbic acid and thioglycollic acid, but not ferrous ion, brought about the irreversible inactivation of the enzyme in the absence of substrate upon incubation at 37°. The time course of inactivation by ascorbic acid and by thioglycollic acid of the casein phosphatase and the ATPase activities of the enzyme is shown in Fig. 2. Within 20 min there was about a 90 % loss of activity in the presence of ascorbic acid. Enzyme in the presence of buffer alone was stable under these conditions. Addition of ferrous ion or more reducing agent or both did not reactivate the enzyme. Addition of fresh enzyme, however, gave full activity proportional to the amount of enzyme added to the mixture. The hydrolysis of all active substrates was abolished to the same extent by this inactivation procedure.

Ferrous ion protection against the ascorbic acid inactivation of the enzyme (Fig. 3)

Ferrous ions, unlike other activators of enzyme activity, did not cause the inactivation of the enzyme during a 20 min preincubation period in the absence of substrate. In fact, ferrous ions, over the range of 10^{-5} to 10^{-3} *M*, effectively protected against the inactivation by ascorbic acid. None of the other metal ions previously listed, with the exception of ferric ion, which is presumably reduced to ferrous ion by ascorbic acid, protected the enzyme.

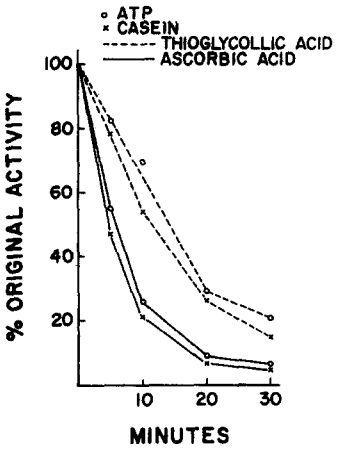

Fig. 2. Enzyme inactivation by reducing agents in the absence of substrate. Each test tube contained in a volume of 0.5 ml, 100 μmoles of acetate buffer of pH 5.8, 5 μmoles of neutralized ascorbic acid or thioglycollic acid and 10 μg of enzyme (specific activity with casein = 5.0). After preincubation at 37° for the time intervals indicated, 0.5 ml containing either 5 μmoles of ATP or 8 μmoles of casein phosphate was added and the incubation continued for 15 min. The reactions were stopped by the addition of 1 ml of cold 20% trichloroacetic acid and the samples were analyzed for inorganic phosphate.

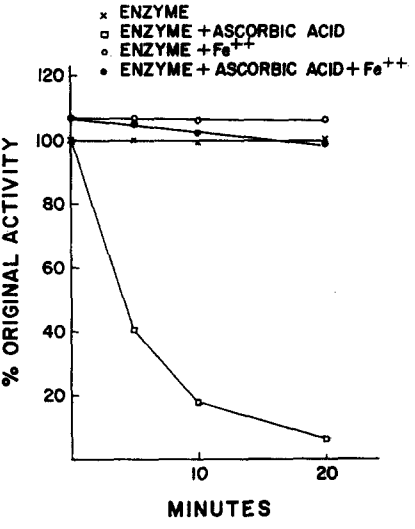

Fig. 3. Ferrous ion protection against ascorbic acid inactivation of casein phosphatase activity. Experimental test tubes contained in a volume of 0.5 ml, 100 μmoles of acetate buffer of pH 5.8, 10 μg of enzyme (specific activity with casein = 5.0) and, as indicated, 5 μmoles of neutralized ascorbic acid and/or 0.5 μmoles of ferrous chloride. After preincubation at 37° for the time intervals indicated, 0.5 ml containing 8 μmoles of casein phosphate was added and incubation continued for 15 min. 5 μmoles of neutralized ascorbic acid were added to the vessels containing enzyme alone for the assay of enzyme activity during the second incubation period. The reactions were stopped by the addition of 1 ml of cold 20% trichloroacetic acid and the samples were analyzed for inorganic phosphate.

TABLE IV

EFFECT OF PREINCUBATION UNDER VARIOUS CONDITIONS ON ENZYME ACTIVITY WITH SEVERAL SUBSTRATES

Experimental test tubes with no preincubation contained in a final volume of 1 ml, 100 μmoles of acetate buffer of pH 5.8, 5 μmoles of substrate, 8 μg of enzyme (specific activity with casein = 6.0) and, where indicated, 5 μmoles of neutralized ascorbic acid and 0.5 μmoles of ferrous chloride. Incubation was at 37° for 15 min. Experimental test tubes which were preincubated contained in a volume of 0.5 ml, 100 μmoles of acetate buffer of pH 5.8, 8 μg of enzyme and, where indicated, 5 μmoles of neutralized ascorbic acid and 0.5 μmoles of ferrous chloride. After 20 min preincubation at 37°, 5 μmoles of substrate in a volume of 0.5 ml were added to each test tube and incubation was continued for 15 min. All reactions were stopped by the addition of 1 ml of cold 20% trichloroacetic acid and analysis for inorganic phosphate was carried out as described in METHODS. Results are expressed as μg of phosphate liberated in 15 min.

Substrate	*No preincubation*			*Preincubation*		
	1 *No reducing agent*	*2* *Ascorbic acid*	*3* *Ascorbic acid + ferrous chloride*	*4* *No reducing agent**	*5* *Ascorbic acid*	*6* *Ascorbic acid + ferrous chloride*
Casein	1.4	21	23	20	0.5	19
ATP	19	125	133	130	11	125
Pyrophosphate**	4.0	20	43	27	1.0	35
Phenylphosphate	13	55	102	80	5.5	95

* Ascorbic acid added after preincubation period for assay of enzyme activity.
** Measured as described in Table II.

Table IV shows the effect of preincubation of enzyme under various conditions on the activity with ATP, pyrophosphate, phenyl phosphate and casein as substrate. Stimulation by ascorbic acid is shown in column 2, a slight further stimulation by Fe^{++} is shown in column 3. Stability of activity on incubation at 37° without ascorbic acid is shown in column 4. Inactivation by ascorbic acid in the absence of substrate is shown in column 5 and protection by ferrous ions against this inactivation is shown in column 6.

Experiments with phosphoproteins

Although the wide range of specificity of the purified spleen enzyme reduces its usefulness as an analytical tool to study phosphoprotein intermediates in various enzyme reactions, some attempts were made to explore the feasibility of this approach. Phosphoglucomutase isolated from rabbit muscle is a phosphoprotein and does not react with glucose-1,6-diphosphate without a suitable phosphate acceptor[21,22]. Action of the phosphoprotein phosphatase on this phosphoprotein was determined by measuring its reactivity with glucose-1,6-diphosphate before and after treatment with the phosphatase. Following incubation of phosphoglucomutase (0.05 μmole) with 50 μg of the purified phosphoprotein phosphatase, addition of glucose-1,6-diphosphate resulted in the formation of glucose-6-phosphate in amounts stoichiometric with phosphoglucomutase. However, a control test tube which contained only the phosphatase activator, ascorbic acid, yielded the same amount of dephosphoglucomutase. Phosphoglucomutase incubated alone in a second control test tube did not accept a phosphate group from glucose-1,6-diphosphate. The interesting possibility that reducing agents, which are known to activate phosphoglucomutase, may labilize the phosphoenzyme was not pursued further. Although a kinetic analysis may have revealed an effect of the phosphatase in addition to ascorbic acid this approach was difficult with such a scarce substrate.

Twice recrystallized hexokinase prepared according to Darrow and Colowick* was found to contain organically bound phosphate (about 1.5 moles/90,000 g). However, spleen phosphoprotein phosphatase did not release this phosphate group. In any case, preliminary experiments on phosphate transfer with hexokinase failed to reveal the formation of a phosphoprotein. A more direct approach to enzymes which are assumed to form a phosphoenzyme in the presence of ATP, by transforming them to ATPases in the presence of a specific phosphoprotein phosphatase was precluded by the rapid cleavage of ATP by the spleen enzyme.

DISCUSSION

The primary purpose of this investigation was to prepare a phosphoprotein phosphatase that could catalyze the dephosphorylation of phosphoenzymes which are assumed to be formed during oxidative phosphorylation and during some other phosphate transfer reactions. This aim was not fulfilled because a separation of the phosphoprotein phosphatase activity from enzyme activity towards ATP and other phosphate esters which participate in phosphate transfer reactions has not been achieved. In fact, further evidence has been obtained that the hydrolysis of ATP

* Unpublished preparation of crystalline yeast hexokinase. We wish to thank Dr. COLOWICK for this information.

and other phosphate esters is catalyzed by the same protein as has been suggested previously[4–6]. Several additional compounds such as phosphorylenol pyruvate, 3-phosphoglyceric acid and fructose-1,6-diphosphate, have been added to the list of hydrolyzable substrates. The relatively rapid cleavage of 3-phosphoglyceric acid as compared with α or β glycerol phosphate points to a large influence of neighboring groups on the susceptibility of the substrate to the enzyme.

In view of these considerations it is not possible to draw any significant conclusions concerning the nature of the phosphate linkage in casein from the fact that all the phosphate groups are cleaved by this enzyme. Nor does the fact that phosphoserine and phosphothreonine are hydrolyzed only slowly eliminate these amino acids as bearers of the phosphate groups in casein, since neighboring peptide linkages and a specific amino acid sequence may profoundly influence susceptibility to hydrolysis[23].

The purification procedure described in this paper results in a highly active soluble phosphoprotein phosphatase of considerable stability. Difficulties in reproducing the original procedure of SUNDARARAJAN AND SARMA[2,3] were traced to the presence of nucleic acids which render the enzyme less soluble. In the course of purification of such preparations smearing of activity was observed, particularly with DEAE cellulose columns from which the nucleoproteins emerged in several rather distinct peaks bearing phosphoprotein phosphatase and ATPase activity of constant ratios. In contrast to the nucleoprotein, the soluble enzyme obtained after treatment with protamine sulfate was not retained by the DEAE cellulose, but was readily absorbed on a cation exchange resin such as Dowex-50. These properties suggest that the free enzyme is a strongly basic protein.

The method of purification described by SINGER AND FRUTON[4] is somewhat simpler than the present one and results in a preparation of approximately the same specific activity. Although for many purposes their method is probably preferable, it should be pointed out that the preparations seemed to differ in several minor aspects and one very critical one. While the perhaps more gentle extraction with salts used in the present work results in an enzyme which is rather inactive in the absence of reducing agents and which is fully activated by ferrous ions, the enzyme preparation of SINGER AND FRUTON obtained by extraction of the spleen at pH 3.0 for 22 h at room temperature is less dependent on reducing agents and is inhibited by ferrous ions. This rather puzzling feature is somewhat reminiscent of the observations of MOKRASCH AND MCGILVARY[24,25] with fructose diphosphatase from liver. Prolonged autolysis at an acid pH, or exposure to proteolytic enzymes, not only increased the activity, but led to alterations in the physical properties of the enzyme. BYRNE[26] also observed that fructose diphosphatase, obtained by mild procedures, cleaves fructose diphosphate at neutrality, in contrast to the enzyme obtained by autolysis at a low pH. It is conceivable that some of the differences observed among protein phosphatases from spleen, such as in the reactivity with creatinephosphate, are due to similar alterations in pH optimum.

Among the minor differences in properties of the protein phosphatase prepared by the two procedures is the cleavage of aliphatic esters, such as 3-phosphoglyceric acid. The hydrolysis of this compound, as well as that of ribulose-1,5-diphosphate and fructose-1,6-diphosphate, in contrast to the much lower reactivity of α or β glycerol phosphate, ribose-5-phosphate, fructose-1-phosphate and fructose-6-phosphate, may be related to the presence of a second acidic group in the more susceptible

substrates. The possibility that these aliphatic phosphate esters are cleaved by a contaminating non-specific phosphatase has not been rigidly ruled out, but it is unlikely because, after inactivation of the phosphoprotein phosphatase activity by short exposure to reducing agents in the absence of substrate, the hydrolysis of these simple esters is also eliminated.

As has been previously observed[2,4], sulfhydryl compounds enhance considerably the activity of beef spleen phosphoprotein phosphatase. With the present preparation the most effective activator was ascorbic acid and, in contrast to other reports, ferrous ions were found to be equally effective. No other metal ion tested stimulated enzyme activity. PAIGEN[19] recently reported the activation of a protein phosphatase obtained from mouse liver by Cu^{++}, Fe^{++} and Ni^{++}. However, the activity was both sulfhydryl and metal dependent, while the spleen enzyme is fully active with ferrous ion alone in the absence of added reducing agents. The concentration of ascorbic acid used in these experiments is not likely to effect the reduction of an S–S group on the enzyme. These observations, together with the relative resistance of enzymic activity to iodoacetate and to *p*-chloromercuribenzoate, cast doubts on the validity of proposed mechanisms of an active site involving an SH group. The inhibition of enzyme activity by metal chelating agents such as 1,10-*o*-phenanthroline, α,α'-dipyridyl and 8-hydroxyquinoline, strongly points to the participation of a metal, probably ferrous ion.

The stability properties of the spleen protein phosphatase are rather unusual. While the activity is preserved after exposure to temperatures up to 70° for prolonged time periods[3,4] the enzyme is rapidly inactivated, even at 37°, in the presence of ascorbic acid or thioglycollic acid. Ferrous ions as well as substrate serve to protect the enzyme against inactivation in the presence of reducing agents. The following hypothesis serves to explain the above findings. The enzyme is isolated from the spleen as a ferric-enzyme complex which has a very low dissociation constant and is of considerable stability to heat. For activity, it is visualized that the ferric-enzyme has to be reduced to form a ferrous-enzyme complex which is more readily dissociable. This ferrous-enzyme complex is stabilized in the presence of substrate. Without substrate the complex dissociates and the metal-less enzyme is rapidly inactivated. Addition of ferrous ions at relatively high concentrations protects the enzyme by simply providing more favorable equilibrium conditions for the maintenance of a ferrous-enzyme complex.

While this manuscript was in preparation, two additional reports on the purification of beef spleen phosphoprotein phosphatase with specific activities two to eight times higher than the present one have been described[6,27]. However, these workers were not concerned with the specific problems of metal activation and substrate specificity dealt with in the present communication.

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