THE INTERCONVERSION OF PHOSPHORYLASE *a* AND PHOSPHORYLASE *b* FROM DOG HEART MUSCLE*

by

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The purification and properties of phosphorylase *a* from rabbit skeletal muscle and the enzymic conversion of phosphorylase *a* to phosphorylase *b* have been extensively studied by G. T. AND C. F. CORI and their collaborators[1-9]. These studies have shown that the enzymic conversion of the *a* to the *b* form yields a product which was inactive when assayed in the absence of adenosine-5-phosphate, but fully active in the presence of adenosine-5-phosphate, and whose molecular weight was approximately one-half that of the *a* form[9]. Recently the enzymic conversion of rabbit muscle phosphorylase *b* to the *a* form, dependent on adenosine triphosphate, has been reported[10,11]. The conversion of the *b* to the *a* form resulted in restoration of enzymic activity in the absence of adenosine-5-phosphate and in an increased sedimentation rate[11].

Studies conducted in this laboratory have shown that incubation of dog liver phosphorylase with liver inactivating enzyme yielded as products inorganic phosphate and a protein (liver dephosphophosphorylase) which was essentially inactive even when assayed in the presence of adenosine-5-phosphate, and which sedimented at the same rate as liver phosphorylase[12,13]. In dog liver slices it was found that radioactive phosphate was incorporated into the enzyme molecule and could be subsequently removed by incubation with liver inactivating enzyme[12]. Furthermore it was shown that both enzymic activity and phosphate could be restored to liver dephosphophosphorylase after incubation with another enzyme fraction from dog liver (dephosphophosphorylase kinase), adenosine triphosphate and magnesium ions[14].

Dog heart muscle phosphorylase provides an example of a phosphorylase from a different tissue which exists in both the *a* (active in the absence of adenosine-5-phosphate) and the *b* (active only in the presence of adenosine-5-phosphate) forms. This report deals with the interconversion of the two forms of heart phosphorylase and with the enzymes obtained from heart muscle which are capable of catalyzing this interconversion.

METHODS

Materials

Glycogen, G-1-P**, Tris, ammonium sulfate, sodium sulfate, calcium phosphate gel, LP,

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** The following abbreviations are used: G-1-P, glucose-1-phosphate; Tris, tris(hydroxymethyl)-aminomethane; LP, liver phosphorylase; dephospho-LP, liver dephosphophosphorylase; IE, inactivating enzyme; AMP, adenosine-5-phosphate; ATP, adenosine triphosphate; HP, heart phosphorylase; phosphokinase, dephosphophosphorylase kinase; TCA, trichloroacetic acid; GSH, reduced glutathione.

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dephospho-LP, and liver IE were prepared as described previously[13,14,15]. AMP and caffeine were purchased from Nutritional Biochemicals Corporation. Disodium ATP was obtained from Pabst Laboratories and Sigma Chemical Company. Crystalline bovine trypsin was purchased from Armour and Company, and crystalline soy bean trypsin inhibitor was obtained from Worthington Biochemical Corporation.

Assay procedures

HP activity was assayed under the same conditions used for LP assay[15]. One unit of enzyme was defined as that amount which caused the liberation of 1.0 mg of inorganic phosphorus in 10 minutes at 37° when the per cent conversion of G-1-P to glycogen was in the range of 12 to 22%.

Heart IE activity was assayed under the same conditions used for the assay of liver IE, using LP as substrate[13]. One unit of inactivating enzyme was defined as that amount which resulted in the inactivation of one unit of LP in 10 minutes at 37°. (One unit of LP as defined elsewhere[15] was approximately equal to 130 phosphorylase units, as defined by CORI *et al.*[3].)

Heart phosphokinase activity was assayed in the same system as that used to assay liver phosphokinase, using dephospho-LP as substrate[14]. When crude fractions were assayed, twice the standard concentration of ATP was used ($2 \cdot 10^{-3} M$ instead of $1 \cdot 10^{-3} M$). One unit of phosphokinase was defined as that amount of enzyme which catalyzed the formation of one unit of LP in 10 minutes at 37°. Specific activity of all three enzymes was expressed as units per milligram of protein.

Preparation of enzymes

(1) *Heart phosphorylase*

Adult dogs were sacrificed in the manner described for the preparation of LP[15]. Before the liver was perfused, the heart was removed, placed in cold 0.2 *M* NaF, allowed to chill for 30 to 60 minutes, blotted and wrapped in aluminum foil, and frozen. For the preparation of HP, hearts stored three to six months at —20° were used, as HP fractions derived from aged hearts appeared to contain less IE contamination than those obtained from fresher hearts. All subsequent operations were carried out at 2° unless specified otherwise.

Step 1. Homogenization and centrifugation. 200 g of frozen hearts (about 2 hearts) were cut into small portions with a chisel and homogenized in 760 ml 0.1 *M* NaF + 40 ml 0.1 *M* K_2HPO_4 for 2½ minutes in Waring blendors. The homogenate was adjusted to pH 6.1 with 1 *N* acetic acid and 50 ml of calcium phosphate gel were added. After stirring for 15 minutes, the homogenate was centrifuged for 20 minutes at 2300 × *g*. The resulting supernatant fluid (heart extract) containing about 80% of the HP, was adjusted to pH 6.5 by the addition of 1 *N* KOH and calcium phosphate gel was again added, using a volume equal to one-twentieth the volume of the extract. After stirring for 15 minutes, the gel was removed by centrifugation at 1000 × *g* and discarded. Most of the IE present was adsorbed on the gel, but small amounts still remained in the supernatant fluid of the gel.

Step 2. Precipitation with alcohol. The supernatant fluid of the gel was adjusted to pH 7.1 with 1 *N* KOH. 33 ml of absolute ethanol at —20° per 100 ml of gel supernatant fluid were added and the mixture was chilled to —5° in a salt-ice bath. The resulting precipitate was collected by centrifugation at —5°, and dissolved in 0.1 *M* NaF using a volume equal to 0.09 the volume of the extract; to the resulting solution was added 0.01 the volume of extract of 0.02 *M* AMP (pH 7.0). The HP was then reprecipitated by the addition of 33 ml of ethanol per 100 ml of fluid used to dissolve the precipitate, collected and dissolved as before. This fraction usually represented about 65% of the phosphorylase in the homogenate with about 10-fold purification. The specific activity of the homogenate averaged 0.12 (+AMP) or 0.10 (—AMP),

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while that of the second alcohol precipitate was 1.5 (+AMP) or 1.1 (—AMP). The second alcohol fraction was frozen and stored 3 to 5 days before use.

Step 3. Reprecipitation with alcohol and collection with ammonium sulfate. After thawing, the second alcohol sample was diluted with 0.5 volumes of 0.1 *M* potassium phosphate buffer at pH 7.4, and was centrifuged for 20 minutes at 7000 × *g*. In some cases the HP tended to adhere to the bulky, inactive precipitate unless the fraction was diluted with buffer. The HP was precipitated by the addition of absolute ethanol to 25% (v/v), collected by centrifugation at —5°, and dissolved in 0.1 *M* NaF (using 0.5 the volume of the supernatant fluid of the diluted, centrifuged second alcohol fraction). The enzyme was precipitated a fourth time with ethanol at 25% (v/v) and dissolved as in the preceding step. The resulting solution was adjusted to 40% saturation by the addition of an equal volume of cold 80% saturated ammonium sulfate (which had been made from ammonium sulfate saturated at room temperature and neutralized with NH_4OH). The precipitate was collected by centrifugation at 8000 × *g* for 20 minutes and dissolved in 5 ml of 0.1 *M* NaF per 100 g of tissue. Occasionally samples were encountered which appeared to contain relatively large amounts of glycogen and in these cases the phosphorylase could not be collected completely unless the ammonium sulfate concentration was increased to 66% saturation. The procedures of Step 3 resulted in an additional 4-fold purification, representing an overall purification from the homogenate of 40-fold with about 40% recovery. Further purification steps have been inconsistent, but occasionally it has been possible to prepare samples with specific activities of over 20 (+AMP).

The first ammonium sulfate fraction was frozen and stored at —20° for 2 to 4 days to further reduce any IE contamination, and was subjected to further ammonium sulfate fractionation. Although no appreciable purification was achieved by these procedures, the fractions so treated did not contain amounts of TCA-soluble inorganic phosphate large enough to interfere with subsequent studies. These preparations were then dialyzed for 15 to 16 hours *vs.* 0.1 *M* NaF and 3 hours *vs.* 3 changes of glass-distilled water before use.

(2) *Heart-inactivating enzyme*

The procedure for the purification of IE from dog heart muscle proved to be identical to that for the liver enzyme[13], except for minor modifications due to the fact that the heart enzyme tended to precipitate at lower ammonium sulfate concentrations than the liver enzyme. To summarize the procedure, an extract was prepared by homogenization and centrifugation in the same manner as for the preparation of HP, except that fresher hearts were used and the addition of calcium phosphate gel to the homogenate was omitted. The IE was adsorbed by the addition of 0.1 volumes of calcium phosphate gel to the extract. The gel was washed, the enzyme was eluted with Na_2SO_4, and after removal of a 0–35% saturation fraction, the IE was collected by precipitation at 65% saturation with ammonium sulfate. The 35 to 65% saturation fraction was fractionated again with ammonium sulfate. An ammonium sulfate solution, saturated at room temperature, was added slowly until a slight haze appeared. This subjective criterion proved more reproducible than adjusting the solution to a defined ammonium sulfate concentration. The resulting precipitate was removed by centrifugation and the IE was collected by adjusting the supernatant solution to 50% saturation with ammonium sulfate.

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This fraction could be stored after lyophilization and was subjected to alcohol fractionation before use[13]. These procedures resulted in over 100-fold purification from the extract with about 10 to 15% recovery of the enzyme.

(3) Dephosphophosphorylase kinase

For best results hearts were used which had been stored at —20° for less than one week.

Step 1. The frozen hearts were homogenized as described for the preparation of HP. The pH of the homogenate was not adjusted and was usually near 7.0. After centrifugation at 2300 × *g* for 30 minutes, the supernatant fluid (extract) was decanted through gauze. To the extract was added 0.15 volumes of absolute ethanol. After chilling to —5°, the resulting mixture was centrifuged at —5°, and the supernatant fluid was discarded. The precipitate was dissolved in a solution containing 0.001 *M* GSH and 0.005 *M* Tris buffer at pH 7.4, using a volume equal to one-fifth that of the extract. The resulting preparation was stored 3 to 5 days at —20° before use. This procedure resulted in an 8- to 9-fold purification from the homogenate with about 70% recovery of the enzyme.

Step 2. The first alcohol fraction was thawed and centrifuged for 20 minutes at 7000 × *g* and the bulky, inactive precipitate was discarded. To the supernatant fluid was added NaCl (6 mg per ml) and 0.15 volumes of absolute ethanol. After chilling to —5°, the precipitate was collected by centrifugation at —5° and was dissolved in a solution containing 0.001 *M* GSH and 0.005 *M* Tris buffer at pH 7.4. This step resulted in an additional 6-fold purification (final specific activity of 75 to 80) with about 90% recovery of the enzyme from the first alcohol fraction. The specific activity of the homogenate averaged 1.6, near that for the corresponding enzyme in dog liver homogenates[14]. By an alternate procedure involving adsorption and differential elution of the enzyme from calcium phosphate gel, it has been possible to achieve a specific activity of over 150.

RESULTS

Conversion of phosphorylase a to b

Extracts of dog heart muscle usually exhibited phosphorylase activity ratios of about 0.8, *i.e.*, the activity in the absence of AMP divided by the activity in the presence of AMP equalled 0.8. During purification, this ratio would become 0.44 to 0.70. When preparations of HP were incubated with purified IE prepared from either dog liver or dog heart muscle, or with crystalline bovine trypsin, the ratio fell to 0.1 or less without appreciably affecting the amount of *b* (+ AMP) activity, as shown in Table I. Aliquots of a dialyzed sample of HP prepared as described in the METHODS section, and containing 0.05 *M* NaCl and 0.01 *M* Tris (pH 7.4), were incubated at 27° with heart and liver IE and with crystalline bovine trypsin until 85 to 90% of the original phosphorylase *a* activity had disappeared. Aliquots of the mixture were removed at various times and diluted in 0.1 *M* NaF for assay (excess crystalline soy bean trypsin inhibitor was added to enzyme dilutions containing trypsin). At the end of the incubation the —AMP/+AMP ratio of all three samples had fallen from 0.62 to below 0.1. Aliquots of the mixtures were then fixed with cold TCA at a final concentration of 8.8%. The precipitated protein was removed by centrifugation and

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TABLE I

THE CONVERSION OF HP *a* TO HP *b* BY HEART AND LIVER IE AND TRYPSIN

30 mg samples of HP containing 4.0 units/mg (+AMP) and 2.4 units/mg (—AMP) were incubated at 27° with 0.18 mg of liver IE, 0.14 mg heart IE, and 0.10 mg of trypsin, respectively, in a volume of 1.1 ml. At the times indicated, 0.7 ml aliquots of the mixtures were removed and 0.15 ml of 50% TCA was added to each sample. Other aliquots were assayed for phosphorylase activity. Excess trypsin inhibitor was added to the remainder of the sample containing trypsin and the remaining portions of the incubation mixtures were later used for reactivation studies.

Enzyme	*Minutes incubated*	*+AMP (units/ml)*	*—AMP (units/ml)*	$\frac{-AMP}{+AMP}$
None	0	107	66.4	0.62
Heart IE	60	120	9.2	0.08
Liver IE	45	106	7.0	0.07
Trypsin	16	105	5.2	0.05

TABLE II

TCA-SOLUBLE INORGANIC PHOSPHATE BEFORE AND AFTER CONVERSION OF HP *a* TO HP *b*

Aliquots of the incubation mixtures used in Table I were fixed with TCA as described in the legend of Table I. After chilling and centrifugation, 0.2 ml and 0.4 ml aliquots of the supernatant solutions were analyzed for inorganic phosphate by a micro modification of the method of FISKE AND SUBBAROW, unless indicated otherwise.

Enzyme	*Units a converted to b per ml*	*TCA-Soluble inorganic P (γ/ml)*	*Inorganic P released (γ/ml)*	*γ P released per unit converted*
None	0	0.564 0.650*	—	—
Liver IE	59.4	1.90	1.34	0.023
Heart IE	57.2	1.79	1.23	0.022
Trypsin	61.2	0.651*	0	0

* Determined by a micro modification of the method of BERENBLUM AND CHAIN.

the supernatant solutions were analyzed for inorganic phosphate by a micro modification of the method of FISKE AND SUBBAROW[16]. A sample of HP containing no added enzymes was treated similarly at the beginning of the incubation period. The results of this experiment are summarized in Table II. It can be seen that approximately 0.02 γ of inorganic phosphorus appeared in the TCA supernatant solution per unit of phosphorylase *a* converted to the *b* form in the samples incubated with either heart or liver IE. Similar results were obtained with an HP sample having a much different purification history and having even less background TCA-soluble phosphate than the sample described in Table II. It was previously observed that 0.3 γ of phosphorus was released when one mg of highly purified liver phosphorylase was completely inactivated by incubation with either liver IE[12,13] or with heart IE (unpublished experiments). When expressed in terms of activity, this figure becomes 0.01 γ of inorganic phosphate released per unit of liver phosphorylase inactivated. The significance of the higher phosphate release from HP is not clear; however, it is probable that the relatively impure HP preparation contained some denatured HP.

It can also be seen in Table II that incubation of HP with trypsin did not result in the liberation of inorganic phosphate. Other experiments have qualitatively sup-

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ported this conclusion, but extracts of trypsin-treated samples sometimes contained material which caused variable results in analyses for inorganic phosphate. Radioactive liver phosphorylase, prepared by incubating dog liver slices with ^{32}P orthophosphate and epinephrine[14], has also been inactivated by brief incubation with trypsin (unpublished experiments). In this case all the radioactivity (and some peptide fragments) became soluble in TCA, but the ^{32}P was not extracted into isobutanol under the conditions of the BERENBLUM AND CHAIN method for the determination of inorganic phosphate[17], indicating that the ^{32}P was present in an organic form.

Conversion of phosphorylase b to a

Aliquots of the incubation mixtures remaining after the removal of aliquots for enzymic assay and phosphorus determinations (Tables I and II) were used to study the conversion of HP *b* to HP *a* by phosphokinase preparations from heart and liver. 0.025 ml aliquots of the incubation mixtures described in Table I were incubated with ATP and phosphokinase in a final volume of 0.5 ml. The complete reaction mixture contained in final concentration $2 \cdot 10^{-2} M$ Tris (pH 7.4), $2 \cdot 10^{-2} M$ NaF, $5 \cdot 10^{-3} M$ $MgSO_4$, $4 \cdot 10^{-3} M$ ATP, and from 6 to 13 units of phosphokinase per 0.5 ml. After 20 to 30 minutes of incubation at 37° in centrifuge tubes, the mixtures were chilled and 2 volumes of a neutralized solution of ammonium sulfate, saturated at room temperature, were added. After chilling to 0°, the samples were centrifuged at 10,000 × *g* for 10 minutes in the cold. The precipitates were washed by suspension in 0.66 saturated ammonium sulfate containing $0.1 M$ NaF, collected by centrifugation, and dissolved in 0.9 ml of cold $0.1 M$ NaF (excess trypsin inhibitor was again added to the samples containing trypsin and trypsin inhibitor). The resulting solutions were assayed for phosphorylase activity and were analyzed for protein.

On incubation with either heart or liver phosphokinase, the samples of HP *b* obtained by incubation with heart or liver IE had been converted to phosphorylase preparations which had $-AMP/+AMP$ ratios of 0.44 to 0.65. This phosphorylase *a* formation required ATP and magnesium ions. Manganous ions were capable of replacing magnesium in the case of heart phosphokinase; however, it was not possible to judge the relative effectiveness of the two metals under the conditions of this experiment. When manganous ions have been substituted for magnesium in the standard assay system employing dephospho-LP as substrate, heart phosphokinase has displayed only 35% the activity observed in the presence of magnesium ions. The metal requirement of the phosphokinase reaction may be significant and merits further investigation.

When samples of HP *b* obtained by incubation with trypsin were incubated with phosphokinase preparations, ATP and Mg^{++}, no formation of phosphorylase *a* was observed. In a parallel experiment with HP *b* made by incubation with IE, mixtures of trypsin and trypsin inhibitor were not inhibitory to the formation of HP *a*. Similar observations have been made on samples of liver phosphorylase inactivated by brief incubation with trypsin, *i.e.*, no reactivation was observed when incubated with liver phosphokinase, ATP, and Mg^{++} (unpublished experiments).

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DISCUSSION

It has been previously reported that the IE from dog liver (and heart muscle) catalyzed the inactivation of LP and that inorganic phosphate was formed during the reaction[12,13]. The results with enzymes from heart provide an example of a phosphorylase which exhibits *a* and *b* forms, in which the conversion of the *a* to the *b* form by heart or liver IE also results in release of inorganic phosphate. It seems likely that in the future the inactivating enzymes may become established as phosphorylase phosphatases, or as phosphatases with even more clearly defined specificities. The protein products resulting from incubation of either LP or HP *a* with either heart or liver IE can be subsequently restored to near their original activity by incubation with either heart or liver phosphokinase and ATP. In the case of desphospho-LP it was possible to demonstrate that the IE-hydrolyzable phosphate, as well as enzymic activity, was restored to the protein after incubation with ATP and liver phosphokinase[14]. Although highly purified HP *a* has not been available in sufficient quantities to allow similar studies, it does not seem unreasonable to surmise that phosphate transfer to HP *b* from ATP occurred during the formation of HP *a* in the presence of phosphokinase. The possibility of molecular weight change associated with the interconversion of HP *a* and HP *b* has not yet been investigated. It is not clear how closely the interconversions of phosphorylases from dog liver and dog heart resemble the interconversion of rabbit muscle phosphorylases catalyzed by the phosphorylase-rupturing enzyme[9] and by extracts of rabbit muscle[10,11] and of lobster tail muscle[18]. However, the requirement for ATP in the conversions: 1. dephospho-LP to LP, 2. HP *b* to HP *a*, and 3. rabbit muscle phosphorylase *b* to *a* invite the deduction that phosphate transfer is involved in all three cases. Furthermore, epinephrine has been shown to accelerate both the conversion of dephospho-LP to LP in liver slices[12,14] and the conversion of phosphorylase *b* to *a* in rat diaphragm[19], which might indicate a similarity in the two reactions involved.

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SUMMARY

1. The purification from dog heart of phosphorylase *a*, a phosphorylase phosphatase and a dephosphophosphorylase kinase has been described.

2. Inorganic phosphate was formed during the conversion of heart phosphorylase *a* to *b* by the phosphorylase phosphatases from heart and liver. No inorganic phosphate formation was detected when this conversion was catalyzed by trypsin.

3. Heart phosphorylase *b*, formed by the action of the phosphorylase phosphatases, could be converted to the *a* form in the presence of ATP, Mg^{++} and the dephosphophosphorylase kinases from either heart or liver. Heart phosphorylase *b*, formed by the action of trypsin, was not converted to the *a* form.

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REFERENCES

1 A. A. GREEN AND G. T. CORI, *J. Biol. Chem.*, 151 (1943) 21.
2 G. T. CORI AND A. A. GREEN, *J. Biol. Chem.*, 151 (1943) 31.
3 C. F. CORI, G. T. CORI AND A. A. GREEN, *J. Biol. Chem.*, 151 (1943) 39.
4 G. T. CORI AND C. F. CORI, *J. Biol. Chem.*, 151 (1943) 57.
5 A. A. GREEN, *J. Biol. Chem.*, 158 (1945) 315.
6 G. T. CORI AND C. F. CORI, *J. Biol. Chem.*, 158 (1945) 321.
7 G. T. CORI, *J. Biol. Chem.*, 158 (1945) 333.
8 C. F. CORI AND G. T. CORI, *J. Biol. Chem.*, 158 (1945) 341.
9 P. J. KELLER AND G. T. CORI, *J. Biol. Chem.*, 214 (1955) 127.
10 E. H. FISCHER AND E. G. KREBS, *Federation Proc.*, 14 (1955) 210.
11 E. H. FISCHER AND E. G. KREBS, *J. Biol. Chem.*, 216 (1955) 121.
12 E. W. SUTHERLAND AND W. D. WOSILAIT, *Nature*, 175 (1955) 169.
13 W. D. WOSILAIT AND E. W. SUTHERLAND, *J. Biol. Chem.*, 218 (1956) 469.
14 T. W. RALL, E. W. SUTHERLAND AND W. D. WOSILAIT, *J. Biol. Chem.*, 218 (1956) 483.
15 E. W. SUTHERLAND AND W. D. WOSILAIT, *J. Biol. Chem.*, 218 (1956) 459.
16 C. H. FISKE AND Y. SUBBAROW, *J. Biol. Chem.*, 66 (1925) 375.
17 I. BERENBLUM AND E. CHAIN, *Biochem. J.*, 32 (1938) 295.
18 R. W. COWGILL AND C. F. CORI, *J. Biol. Chem.*, 216 (1955) 133.
19 E. W. SUTHERLAND, *Ann. N.Y. Acad. Sci.*, 54 (1951) 693.

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