THE PHOSPHORYLASE *b* TO *a* CONVERTING ENZYME OF RABBIT SKELETAL MUSCLE*

by

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As a result of the extensive studies of CORI AND CORI on the regulatory mechanisms of glycogen metabolism, the concept was established that phosphorylase exists in two interconvertible forms, *a* and *b*, in skeletal muscle. The conversion of phosphorylase *a* to *b* was shown to be due to the action of an enzyme designated as the PR enzyme[1,2]. In a previous paper from this laboratory, it was shown that phosphorylase *b* in fresh rabbit muscle extracts could be converted to phosphorylase *a* during a short period of incubation in the presence of certain added divalent metal ions and ATP**. This reaction has been studied in an isolated system with purified components, and a method for assaying the converting enzyme*** has been developed. Phosphorylase *a* produced from phosphorylase *b* in the presence of ^{32}P-ATP has been isolated and found to contain firmly bound isotopic phosphate.

EXPERIMENTAL AND RESULTS

Preparation of phosphorylase b

Muscle extracts were prepared by the method of ILLINGWORTH AND CORI[4] except that extractions were carried out at 25° rather than in the cold room. In addition, three extractions were made, the first and second with one volume of distilled water each and the third with a half volume. The combined extracts§ were filtered through cotton, but were not filtered through paper, since this latter procedure could ordinarily contribute sufficient metal ions to cause phosphorylase *a* formation[3]. The extract was dialyzed against cold distilled water for 3 ½ hours§§, after which the pH was adjusted to 6.0, and the precipitate which formed was removed by centrifugation. (This precipitate was saved for the preparation of converting enzyme.) Phosphorylase was precipitated from the supernatant solution at 0.41 saturation of ammonium sulfate, as described by ILLINGWORTH AND CORI, and collected by centrifugation. The packed

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** The following abbreviations will be used herein: ATP, adenosine triphosphate; AMP, adenosine-5'-phosphate; GP, glycerophosphate; EDTA, ethylenediaminetetraacetate; Tris, tris-(hydroxymethyl)-aminomethane.

*** The enzyme catalyzing the conversion of phosphorylase *b* to *a* will be referred to temporarily as the converting enzyme.

§ Fraction 1 in Table I.

§§ Fraction 2 in Table I.

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precipitate was dissolved in sufficient water to give a protein concentration of approximately 4%*.

At this stage the phosphorylase *b* preparation was always found to contain traces of converting enzyme, but the following treatment served to inactivate this contaminant completely. The solution was dialyzed at 3° for 18 hours against several changes of 0.001 *M* Tris buffer, pH 7.5. Neutral 0.1 *M* EDTA and 0.3 *M* cysteine solutions were added to the dialyzed protein solution to final concentrations of $3 \cdot 10^{-4}$ *M* and $1.5 \cdot 10^{-2}$ *M*, respectively. The pH was adjusted to 8.8 by addition of 2 *M* Tris, and the mixture was incubated at 37° for 1 hour. The solution was readjusted to pH 7.5 with 1 *N* acetic acid and stored at 3°.

In a typical preparation starting with 510 g of muscle, the yield of phosphoralyse *b* in the final solution was 1,650,000 units** with a specific activity of 1,200 units per mg protein. The electrophoretic pattern obtained in 0.1 μ phosphate buffer, pH 7.3, showed the presence of several components, but one of these comprised 70% of the total area; the mobility of this component was $-3.1 \cdot 10^{-5}$ cm^2 volt^{-1} sec^{-1}, which compares closely with the value shown by GREEN[6] for phosphorylase *b* under these conditions. The sedimentation pattern in the ultracentrifuge (Fig. 1) showed a major peak with an $S_{20,w}$ equal to 8.65 Svedberg units; KELLER AND CORI[7] reported a value of 8.2 for the sedimentation constant of phosphorylase *b*.

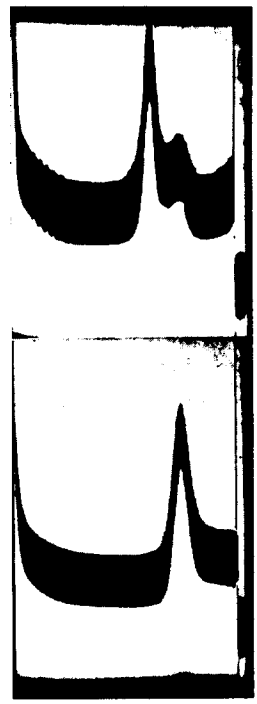

Fig. 1. Changes in the ultracentrifuge pattern during conversion of phosphorylase *b* to *a*. Centrifugations were carried out for 36 minutes in the Spinco ultracentrifuge, operating at 50,740 r.p.m., in a 0.06 *M* GP-0.03 *M* cysteine —0.1 *M* KCl buffer, pH 6.8. Upper photograph: Phosphorylase *a* obtained in conversion reaction. Lower photograph: Phosphorylase *b* preparation used as substrate in conversion reaction (see text).

In some preparations, PR enzyme was present as a troublesome contaminant. It is of considerable interest that the type of PR enzyme activity present in this phosphorylase *b* fraction was activated strongly by Mn^{++} ions. CORI AND CORI[1] originally reported that the PR enzyme was activated by this metal, but KELLER AND CORI[2] later found that their purified enzyme was inhibited by Mn^{++} ions.

Purification of the converting enzyme

The precipitate obtained after dialysis and pH adjustment of the rabbit muscle extract (see above) was suspended immediately in 20 ml of water, brought to pH 7.5 with 0.2 *M* $NaHCO_3$ solution, and stirred thoroughly to break up all visible particles***. It was frozen and stored§ at –20°. The suspension was thawed at 15°, and centrifuged at 18,000 *g* for 30 minutes in the cold. The moderately turbid

* For a rabbit of average size, yielding 500 g of excised muscle, approximately 25 ml of water is used.

** Phosphorylase activities were determined according to the method of ILLINGWORTH AND CORI[4]. Protein concentrations were determined by the method of ROBINSON AND HOGDEN[5].

*** Fraction 3 in Table I.

§ Ordinarily the preparation was continued the next day. It is possible, however, to leave the material at —20° for at least two months without loss of activity.

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supernatant solution* was brought to 0.33 saturation with saturated ammonium sulfate solution (25°), pH 6.8, and centrifuged after standing for 15 minutes. The precipitate was dissolved in 20 ml of a 5% sucrose — 0.1 *M* phosphate buffer, pH 7.5** and centrifuged for 60 minutes at 40,000 r.p.m. in the Spinco, Model L, preparative ultracentrifuge (approximately 100,000 *g*). The clear solution was decanted and stored at —20°***.

Table I shows the recovery and activity of converting enzyme at various steps of the procedure. It will be noted that a large proportion of the total units was lost in the dialysis of muscle extract, but part of this activity seemed to reappear in subsequent steps. The over-all recovery was 33% with a 65-fold purification.

TABLE I

PURIFICATION OF CONVERTING ENZYME

The stages in the preparation at which the fractions were obtained are indicated in footnotes. (For Fractions 1 and 2, see "Preparation of phosphorylase *b*".) Units of converting enzyme are defined in the text.

Fraction	*Volume ml*	*Protein mg/ml*	*Total activity units* $\times 10^{-3}$	*Specific activity Units/mg*
1. Crude muscle extract	1,040	15.8	1,103	67
2. Dialyzed extract	1,060	15.2	419	26
3. Precipitate obtained at pH 6.0 taken up in bicarbonate	33	40.0	561	425
4. Supernatant solution after centrifugation	26	20.2	504	960
5. 0.33 ammonium sulfate precipitate in sucrose-phosphate	22	8.9	447	2,280
6. Supernatant solution after centrifugation at 100,000 *g*	20	4.1	365	4,450

Conversion of phosphorylase b *to* a

When phosphorylase *b* and converting enzyme, prepared as described above, are incubated in an appropriate buffer in the presence of Mn^{++} ions and ATP, a rapid conversion to phosphorylase *a* occurs. Fig. 2 shows the course of this reaction at four different concentrations of converting enzyme. Curves A–D show the increase in phosphorylase activity as measured in the absence of AMP§.

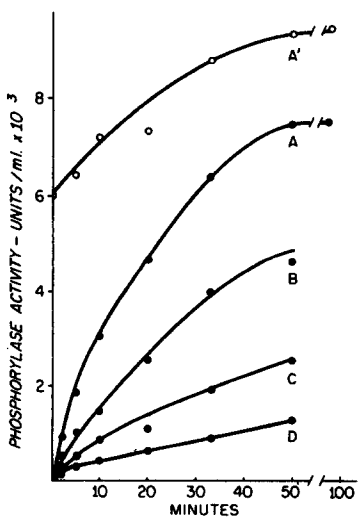

Fig. 2. Conversion of phosphorylase *b* to *a* with varying concentration of converting enzyme. Reaction mixtures were made up as described in the text for the activity test. The phosphorylase *b* solution used contained 38.0 mg protein per ml with a specific activity of 790 units/mg. Concentrations of converting enzyme were 0.04 mg per ml in the reaction mixture of Curve A and A′, 0.02 mg per ml in B, 0.01 mg per ml in C, and 0.005 mg per ml in D. Phosphorylase activities were determined at 1 to 40 and 1 to 100 dilutions of the conversion reaction mixtures. Activities in A–D were determined in the absence of AMP. Activity in A′ was determined in the presence of AMP.

* Fraction 4 in Table I. ** Fraction 5 in Table I.
*** Fraction 6 in Table I. § See footnote ** p. 153.

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Curve A′ shows that a significant increase in activity also occurred as measured in the presence of AMP. The conversion reaction mixture in the experiment of Curves A and A′ was identical and contained the highest concentration of enzyme used in the experiments of Fig. 2. In this case, the reaction seemed to reach completion as indicated by the levelling off that occurred after 50 minutes. At this point the ratio of phosphorylase activity (—AMP) to phosphorylase activity (+AMP) was 0.8. In some experiments (not illustrated) ratios as high as 1.0 have been noted after conversion; this variability is unexplained, but the possibility that activities (—AMP) were being influenced by free AMP carried over into the phosphorylase activity assay system has been excluded.

Converting enzyme activity

In the early part of the conversion reaction the amount of phosphorylase activity (—AMP) formed in a given time is proportional to the amount of converting enzyme added. On this basis, a unit of enzyme activity is defined as the amount of enzyme that gives rise to the formation of 100 units of phosphorylase activity (—AMP) per ml of reaction mixture in 5 minutes at 30°. Reaction mixtures are made up as follows: 0.4 ml 0.125 *M* Tris —0.125 *M* GP buffer, pH 7.8; 0.2 ml phosphorylase *b* solution containing at least 25,000 units per ml; 0.2 ml converting enzyme diluted in neutral 0.03 *M* cysteine solution; and after several minutes incubation at 30°, 0.2 ml $5 \cdot 10^{-3} M$ $Mn(Ac)_2$ — $5 \cdot 10^{-3} M$ ATP* solution, pH 7.8, to start the reaction. Aliquots are removed at 5 minutes and diluted in 0.04 *M* GP — 0.03 *M* cysteine buffer, pH 6.8, for assay of phosphorylase activity according to the method of ILLINGWORTH AND CORI[4]. This dilution prior to the phosphorylase assay stops the conversion reaction. Amount of converting enzyme assayed should not exceed an amount causing conversion of more than one-third of the total phosphorylase *b* present.

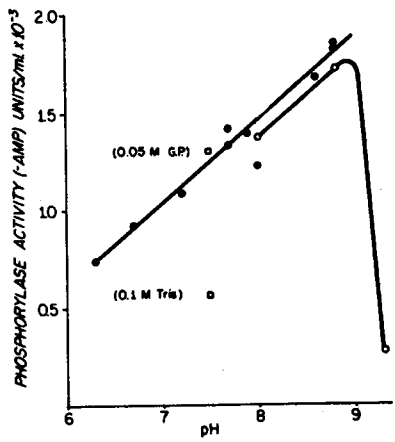

Fig. 3. Converting enzyme activity with varying pH. The composition of the reaction mixtures was the same as described in the text, except for variation in the type of buffer. Points with closed circles indicate 0.025 *M* GP–0.025 *M* Tris buffer. Points with open circles indicate 0.025 *M* GP–0.025 *M* glycine buffer. The buffers for two points at pH 7.5, indicated as open squares, are given in the figure. Concentration of converting enzyme was 0.01 mg per ml in all reaction mixtures.

pH Optimum for converting enzyme

A simple determination of the pH optimum for the phosphorylase *b* to *a* reaction was difficult, because of a marked discontinuity in activities that occurred in changing from one buffer to another. In glycerophosphate the activity increased in a regular manner as the pH was raised from 6.0 to 7.5, the upper limit for this buffer. At this pH, when Tris was substituted for GP, a marked drop in activity occurred (Fig. 3), although this did not happen when a combination of GP and Tris was used. In this mixed buffer,

** Phosphorylase activity, as measured in the absence of AMP, will be indicated as activity (—AMP); when measured in the presence of AMP, as activity (+AMP).

* Crystalline disodium ATP, obtained from Pabst Laboratories, Milwaukee, Wisconsin.

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the activity increase with increasing pH was continuous, as shown in Fig. 3. To reach a pH above 9.0, a GP-glycine buffer was used, and at pH 9.3 the rate was found to be reduced. These effects have not as yet been studied in detail; they suggest an interaction of the buffer ions with components of the reaction mixture. It is known[8], for example, that Tris interacts with Mn^{++}, and, as will be shown in the next section, it appears that the concentration of this component is critical.

Influence of Mn^{++} and ATP concentrations on converting enzyme activity

The metal ion specificity for the reaction using purified components, as in the present study, is different from what was found[3] for the conversion occurring in crude muscle extracts. In the latter case Mn^{++} and Ca^{++} were active and Mg^{++} was inactive, whereas in the purified system Mn^{++} and Mg^{++} are active, but Ca^{++} is completely inactive. It would appear likely that the Ca^{++} effect in crude extracts was an indirect one, and the failure of Mg^{++} to activate under these circumstances was due to its non-specific binding. These points are being investigated further.

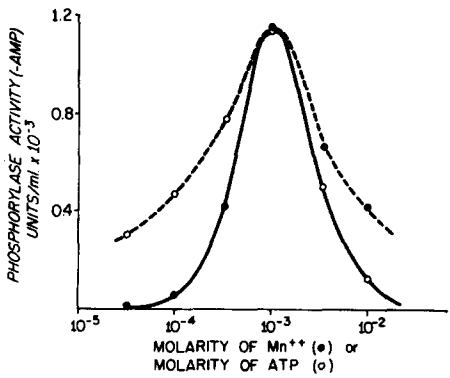

Fig. 4. Effect of variation in the ratio of Mn^{++} and ATP on conversion rates. Reaction mixture as described for the activity test, except for Mn^{++} and ATP. Converting enzyme concentration = 0.01 mg/ml. Closed circles indicate variation of [Mn^{++}] with ATP constant at $10^{-3}M$. Open circles indicate variation of [ATP] with Mn^{++} constant at $10^{-3}M$. Points connected by solid line on either side of maximum correspond to [ATP]/[Mn^{++}] > 1. Points connected by dotted line correspond to [Mn^{++}]/[ATP] > 1.

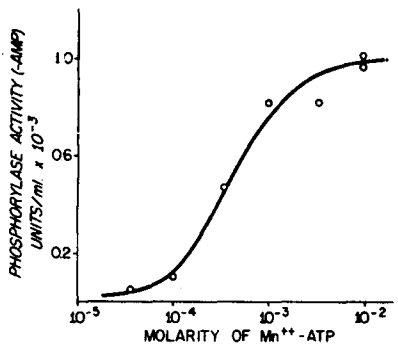

Fig. 5. Effect of variation in concentration of Mn^{++}-ATP complex on conversion rates. Reaction mixtures as in Fig. 3, except the ratio of [Mn^{++}] to [ATP] constant and equal to 1. Total concentration varied.

A study of the effect of variation in the concentration of Mn^{++} and ATP on the reaction showed (Fig. 4) that maximum activity was obtained when these two components were present in a 1 to 1 ratio*. This result is consistent with the interpretation that a monomanganous-ATP complex is active in the reaction, and that other complexes which might be formed[9] containing a higher number of Mn^{++} per ATP, or ATP per Mn^{++}, are inactive or inhibitory. Fig. 5 shows the variation in activity with varying concentrations of a 1:1 mixture of Mn^{++} and ATP; half maximum velocity was at $4 \cdot 10^{-4}M$. In all these experiments the Mn^{++}-ATP mixture was added as the last component to minimize the effects of interaction of cysteine with Mn^{++}.

* At very low concentrations of the components, *i.e.*, $10^{-4}M$ or less, an excess of Mn^{++} over ATP is equired for maximum activity. This is probably due to complexing of the metal ion by substances other than ATP.

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Conversion of phosphorylase b *to* a *in the presence of* ^{32}*P-ATP*

In order to determine the role of ATP in the conversion of phosphorylase *b* to *a*, the reaction was carried out in the presence of ^{32}P-ATP*. In one experiment, the conversion was carried out in a dialyzed crude muscle extract[3] and phosphorylase *a* was isolated, essentially according to the Cori method[4] and treated with Norit A (Pfanstiehl) to remove adsorbed nucleotides. In a second experiment, purified phosphorylase *b* was used with purified converting enzyme, as described earlier in this paper, except on a much larger scale. After conversion, the reaction mixture was brought to 0.41 saturation with ammonium sulfate, and the precipitated protein was crystallized and recrystallized as in the Cori method. For counting, the protein samples in both experiments were precipitated in 5% trichloroacetic acid, washed several times with the acid, and then taken up in 3% NaOH and plated.

Table II shows the results of the two experiments. In Expt. 1, it would appear that there was close to one mole of phosphate incorporated per mole** of phosphorylase *a*, if the isolated sample were considered as pure enzyme. On the basis of 2,500 units per mg as the specific activity of pure phosphorylase *a*[10], the enzyme in this experiment was only 75% pure, and the moles of phosphate per mole of pure enzyme would be 1.6. In the second experiment, showing somewhat greater incorporation per mg of protein, although the sample appeared to be well crystallized, its specific phosphorylase activity was very low for unexplained reasons and could scarcely serve as a useful value for calculations. This sample, when analyzed in the ultracentrifuge (Fig. 1), was found to contain 74% of a component with an $S_{20,w} = 13.8$ corresponding to phosphorylase *a*[7]. On this basis the moles of ^{32}P incorporated per mole of phosphorylase *a* were calculated to be 2.2.

TABLE II

UPTAKE OF ^{32}P DURING PHOSPHORYLASE *a* FORMATION IN THE PRESENCE OF ^{32}P-ATP

Expt. 1. Phosphorylase *a* isolated after formation from phosphorylase *b* in dialyzed crude muscle extracts[3] in the presence of 10^{-4} *M* ^{32}P-ATP ($1.265 \cdot 10^6$ c.p.m. per μM ATP-terminal phosphate) and 10^{-3} *M* Mn^{++}.

Expt. 2. Phosphorylase *a*, isolated and recrystallized after formation from purified phosphorylase *b* and converting enzyme in the presence of 10^{-4} *M* ^{32}P-ATP ($2.850 \cdot 10^5$ c.p.m. per μM ATP-terminal phosphate) and 10^{-3} *M* Mn^{++}.

Expt.	*Phosphorylase activity (+AMP) units/mg*	*Ratio* $\frac{\text{Activity (-AMP)}}{\text{Activity (+AMP)}}$	*c.p.m. per mg protein*	*Moles ^{32}P incorporated per $4.95 \cdot 10^5$ g protein*
1	1,890	0.70	3,035	1.2
2	1,245	0.50	940	1.6

DISCUSSION

The finding of an enzyme that catalyzes the phosphorylase *b* to *a* conversion in a cell-free system, now makes it possible to study the interconversion of these two

* Two samples of ^{32}P—ATP were used; one kindly furnished by Dr. ARTHUR KORNBERG, to whom we are greatly indebted, the other purchased from Schwarz Laboratories, Inc., Mt. Vernon, New York.

** The molecular weight of phosphorylase *a* is 495,000[7].

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forms. Separate enzymes are required for the reaction in each direction. The purified *b* to *a* converting enzyme, as described in this paper, shows no PR enzyme activity; it has also been determined that the PR enzyme, as purified by KELLER AND CORI[2], is free of *b* to *a* converting activity[3]. It is to be expected that the availability of both of these enzymes will facilitate a study of the structural differences and properties of the two forms of phosphorylase. For example, no explanation is available for the role of adenylic acid in the phosphorylase system; this question, as well as the possibility of a prosthetic group[11,12] in phosphorylase *a* requires investigation. In addition, no structural explanation is at hand for the two-fold difference in the molecular weight[7] of phosphorylase *a* and *b*.

The experiments with ^{32}P-labeled ATP have shown conclusively that isotopic phosphorous becomes incorporated into phosphorylase *a* formed in the conversion reaction; it is present in a form that is not split off by trichloracetic acid treatment. As described earlier, the results do not permit a definite conclusion as to the exact number of ^{32}P atoms incorporated per mole of phosphorylase *a*, but it would appear that there are at least two. Further experiments with more highly purified and stable phosphorylase *b* will be required to clarify this point. Other proteins, including serum albumin, α-lactalbumin, β-lactoglobulin, *B. subtilis* α-amylase, and yeast glyceraldehyde 3-phosphate dehydrogenase were not phosphorylated in the presence of ^{32}P-ATP and the converting enzyme.

Attempts to determine whether or not adenosine diphosphate (ADP) is a product in the *b* to *a* reaction have been rendered difficult by the presence of contaminating enzymes in the phosphorylase *b* preparation which are known to act on ATP independently of the conversion. These side reactions become apparent when the phosphorylase *b* concentration is raised to the high level required to approach the molarity of ATP. If ADP is a product, then it would appear reasonable to think of the *b* to *a* conversion as a typical kinase reaction in which the terminal phosphate of ATP is transferred to a specific protein substrate (phosphorylase *b*). Experiments (not illustrated) have shown that ^{32}P is released when ^{32}P-labeled phosphorylase *a* is converted to phosphorylase *b* by the PR enzyme; but the form in which the isotope is released has not been determined. CORI AND CORI[1] reported a release of organic phosphate when phosphorylase *a* was converted to phosphorylase *b*. Working with liver slices, SUTHERLAND AND WOSILAIT determined that inorganic phosphate is released when liver phosphorylase is inactivated; phosphate is taken up when the phosphorylase is reactivated[13].

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SUMMARY

A method for the purification of the enzyme catalyzing the conversion of phosphorylase *b* to *a* is described. After a 65-fold increase in specific activity, the enzyme obtained is free of PR enzyme activity.

The course of the reaction at several concentrations of converting enzyme is illustrated, and converting enzyme units are defined. The optimum pH for the enzyme is approximately 9.0; the reaction requires Mn^{++} or Mg^{++} ions and ATP. It is shown that a mono-manganous-ATP complex is probably acting in the reaction.

Conversion of phosphorylase *b* to *a* is carried out in the presence of ^{32}P-ATP, and an incorporation of at least 2 moles of ^{32}P per mole of phosphorylase *a* is found to occur.

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