Biochimica et Biophysica Acta, 1000 (1989) 297–301
Elsevier

Commentary by

Edmond H. Fischer and Edwin G. Krebs

Howard Hughes Medical Institute, University of Washington, Seattle, WA (U.S.A.)

on 'The phosphorylase *b* to *a* converting enzyme of rabbit skeletal muscle"
by E.G. Krebs and E.H. Fischer
Biochim. Biophys. Acta 20 (1956) 150–157

Edwin Krebs (left) and Edmond Fischer

The background for our work on the glycogen phosphorylase *b* to *a* 'converting enzyme' goes back to research carried out by Carl and Gerty Cori, together with Arda Green, on the role of adenylic acid in controlling the activity of phosphorylase from rabbit skeletal muscle. These workers had found that this enzyme exists in two forms, which they designated as phosphorylase *b* and phosphorylase *a* [1,2]. Phosphorylase *b*, which required relatively high concentrations of 5′-AMP for activity, was considered to be a physiologically inactive form of the enzyme, whereas phosphorylase *a*, which exhibited almost full activity in the absence of this cofactor, was thought of as the active species. Phosphorylase *a* was isolated as a crystalline enzyme and partially characterized. It was thought likely that phosphorylase *a* might contain AMP as a prosthetic group that was lacking in phosphorylase *b*. An enzyme, originally referred to as the PR (prosthetic group removing) enzyme, was discovered, which catalyzed the conversion of phosphorylase *a* to *b* in vitro. The Coris were unable to demonstrate any conversion of phosphorylase *b* to phosphorylase *a* in vitro, but they did obtain evidence that it probably occurs in vivo. On the basis of their experiments, the Coris concluded

0006-3002/89/$03.50

that the form of phosphorylase that predominates in resting skeletal muscle is phosphorylase *a* and that muscle contraction leads to the formation of phosphorylase *b*. In retrospect, it is difficult to rationalize a satisfactory physiological basis for this concept, since glycogen breakdown catalyzed by active phosphorylase is needed to supply energy for muscle contraction, and we now known that conversion of phosphorylase *a* to *b* would work against this process. It should be remembered, however, that in 1945 it was believed that phosphorylase also catalyzed glycogen synthesis, and the activity state of this enzyme would not necessarily be considered as important in determining the direction of the reaction.

In the late 1940's and early 1950's, experiments by Earl Sutherland, carried out initially in the Coris' laboratory, showed that liver glycogen phosphorylase appeared to shuttle back and forth between inactive and active states within the cell. Work carried out on liver slices supported the concept that epinephrine and glucagon cause conversion of the inactive form to the active form [3]. It was also shown [4] that epinephrine caused conversion of phosphorylase *b* to *a* in the muscle diaphragm.

Our own work on glycogen phosphorylase was started in 1954 and was not directed initially toward elucidating the biochemical mechanism of the interconversion reactions. Instead, we were interested in the possible role of AMP in the activation process and later became intrigued by a report that this enzyme might contain a uridine nucleotide as a prosthetic group. The first step was to isolate pure crystalline phosphorylase *a* by the Cori procedure, but we were completely unsuccessful in our initial efforts. Instead of isolating phosphorylase *a*, we obtained only phosphorylase *b*, and the latter form would not crystallize, a step that was essential for obtaining pure protein. A careful comparison of what we were doing with the published isolation procedure [5] revealed that we had substituted a high-speed centrifugation step for filtration through paper as a means of clarifying the crude skeletal muscle extract. When the filtration step was included, phosphorylase *a* was obtained. The different result was due to the fact that filter paper had introduced calcium ions and triggered the conversion of phosphorylase *b* to *a* in vitro [6]. The form of phosphorylase present in 'resting' skeletal muscle was apparently phosphorylase *b* and not *a* as had been believed by the Coris [7]. In this initial work we established that ATP is required for the conversion of phosphorylase *b* to *a* and that a separate protein fraction, a 'converting enzyme', was needed for the reaction. Although the molecular mechanism by which the introduction of Ca^{2+} caused this conversion in the extract was not immediately apparent to us, it was eventually determined that it was due to an intrinsic requirement for a divalent metal ion by the 'converting enzyme' and to its proteolytic activation by a Ca^{2+}-dependent proteinase [8].

After demonstrating the conversion of phosphorylase *b* to *a* in muscle extracts, our next goal was to establish a quantitative assay for the convering enzyme so that it could be purified and the reaction kinetics established. This is the subject that was addressed in the article that is being reprinted in this volume. In order to develop such an assay it was essential that each of the two proteins involved, i.e., phosphorylase *b* and the converting enzyme (we refrained from calling it phosphorylase kinase at that time, because we had not yet established that ADP was a product) be purified sufficiently so that they were no longer contaminated with each other. This was done, and it was gratifying to see that the phosphorylase *b* to *a* reaction could then be studied in much the same way that one would study any other kinase (such as hexokinase). We were able to perform elementary kinetic studies, characterize the metal requirement, and even make a stab at determining the stoichiometry of phosphorylation, thanks to the generosity of Arthur Kornberg, who supplied us with [γ-^{32}P]ATP. Most of our measurements were carried out by following the changes in phosphorylase activity in the presence and absence of AMP, since the characteristic activity patterns of phosphorylase *b* (requires AMP) and phosphorylase *a* (relatively independent of AMP) were well known [1].

In a subsequent study [9] it was determined that the nucleotide product of the *b* to *a* reaction was, indeed, ADP. The stoichiometry was established with much greater accuracy than in the preliminary study, and the amino-acid sequence at the phosphorylation site was determined [10]. Finally, it was shown that the product of the inactivating enzyme (PR enzyme) was inorganic phosphate. Thus, by 1959 it had become possible to write specific equations for the interconversion reactions:

$$\underset{\text{(dimeric)}}{\text{phosphorylase } b} + 4\text{ATP} \xrightarrow[\text{kinase}]{\text{phosphorylase}} \underset{\text{(tetrameric)}}{\text{phosphorylase } a} + 4\text{ADP}$$

$$\underset{\text{(tetrameric)}}{\text{phosphorylase } a} + 4\text{H}_2\text{O} \xrightarrow[\text{(PR enzyme)}]{\text{phosphorylase phosphatase}} 2 \underset{\text{(dimeric)}}{\text{phosphorylase } b} + 4\text{P}_\text{i}$$

While a single serine residue is phosphorylated in this particular substrate, in later years this would prove to be the exception rather than the rule. Most proteins are phosphorylated at multiple sites, with one site sometimes affecting the susceptibility of another to phosphorylation or dephosphorylation.

It should be noted that, during the same period of time that our work on the muscle phosphorylase system was proceeding, a similar track was being followed independently in Earl Sutherland's laboratory involving liver phosphorylase. The first clue that these workers

had that the interconversion of the two forms of phosphorylase might involve phosphorylation-dephosphorylation came with their finding that inorganic phosphate was released when active liver phosphorylase was incubated with a separate liver fraction [11]. They went on to show that the latter fraction contained a phosphorylase phosphatase. Both of the interconversion reactions of liver phosphorylase were demonstrated to occur in cell free systems. An epochal finding that grew out of their studies was the discovery of cyclic AMP (cAMP), which, by an unknown mechanism, was found to shift the balance between nonactivated and activated liver phosphorylase in crude liver fractions containing both of the interconverting enzymes. Sutherland generously supplied us with samples of cAMP, which assisted us in determining that its action was directed toward the activation of phosphorylase kinase [12].

By the early 1960's it was known that in addition to interconversion of phosphorylase *b* and *a* by phosphorylation-dephosphorylation, two other enzymes of glycogen metabolism were regulated by a similar process. One of these was phosphorylase kinase, which, like phosphorylase itself, was found to be activated by phosphorylation and inactivated by dephosphorylation. The other, glycogen synthase, was shown by Larner and his co-workers [13] to be inactivated by phosphorylation and activated by dephosphorylation. Since all of these enzymes are involved in glycogen metabolism, many investigators wondered at that time whether the phosphorylation and dephosphorylation of enzymes might be restricted to this field. In the late 1960's, however, it was recognized in Lester Reed's laboratory that pyruvic dehydrogenase is also regulated by phosphorylation [14]. Thus, the phenomenon could no longer be viewed as being restricted to glycogen metabolism. At about this same time, it was shown that the enzyme that catalyzes the phosphorylation and activation of phosphorylase kinase is a cAMP-dependent protein kinase with a fairly broad specificity [15]. It was determined, for example, that protamine could also be phosphorylated by this enzyme and that glycogen synthase kinase and the cAMP-dependent protein kinase were one and the same [16]. The cAMP-dependent protein kinase was found to be distributed widely in nature, and it was hypothesized that all of the actions of cyclic AMP might be mediated by this kinase [17]. By 1970, the stage was thus set for what turned out to be a period of explosive growth in protein phosphorylation studies – growth that shows no signs of tapering off. A small international society on Metabolic Interconversion of Enzymes was formed and had its first meeting in that year. In a 3-day conference, it was possible to hear reports on *all* of the enzymes that were known to be regulated by phosphorylation-dephosphorylation. Nowadays, the holding of any meeting that would cover all aspects of protein phosphorylation would be completely impossible.

As the pace of research on protein phosphorylation quickened during the 1970's, investigators began to question why it was 'necessary' to have two broad systems for controlling enzyme activity, i.e., allosteric regulation through the reversible binding of ligands and reversible covalent modification such as phosphorylation-dephosphorylation. The question was particularly pertinent in reference to substrates such as phosphorylase and glycogen synthase, since each of them was known to be subject to regulation by positive and negative allosteric effectors. Moreover, it was clear that allosteric and covalent regulation probably worked through similar conformational changes in both of these proteins. However, a basic difference between these two types of regulation soon became apparent. While allosteric control generally reflects intracellular conditions (energy charge or redox potential of the cell, fluctuations in metabolic intermediates, etc.), covalent regulation responds mainly to extracellular signals.

Hormones or growth factors, acting through their specific membrane receptors, often cause the release of various second messengers that affect the activity of protein kinases or phosphatases. The kinases or phosphatases in turn, either directly or through a cascade of phosphorylation-dephosphorylation reactions, ultimately affect target enzymes leading to physiological responses. Cascade systems allow for an enormous amplification as well as fine modulation of an original signal, as discussed in detail by Stadtman and Chock [18]. Furthermore, because different regulatory proteins are involved in these consecutive sets of reactions, and any one of them might be responsive to particular effectors, the coordinate control of separate metabolic pathways can be achieved. For instance, the early work on phosphorylase kinase revealed that this enzyme was under both hormonal and neural regulation. On the one hand, it was subject to hormonal control by phosphorylation-dephosphorylation through the cAMP-dependent protein kinase; and on the other hand, it was also under neural regulation because of its absolute requirement for Ca^{2+}, another cellular second messenger. This provided an explanation for the synchronous regulation of muscle contraction and glycogenolysis, ultimately leading to the synthesis of ATP needed to maintain contraction [19].

The importance of protein phosphorylation-dephosphorylation in controlling cellular processes became more apparent when it was established that a single multifunctional enzyme, the cAMP-dependent protein kinase, could bring about the coordinate control of enzymes in related, albeit different, areas of metabolism such as the pathways of carbohydrate, lipid and steroid metabolism. It was found that biodegradative pathways are stimulated by protein phosphorylation reactions catalyzed by the cAMP-dependent protein kinase whereas the biosynthetic (storage) pathways are in-

hibited by this process. This relationship, first disclosed by the work on phosphorylase and glycogen synthase, has been stressed by Philip Cohen [20,21]. Coordinate control by enzyme phosphorylation is similar in principle to the coordinate regulation of a cluster of functionally regulated genes that are part of a single operon in bacteria. Here again, one signal can generate an overall response by acting at many levels. Coordinate control also occurs as a result of allosteric regulation, but often not at such a fine level of discrimination as occurs in protein phosphorylation systems. Covalent control, mediated by quite specific regulatory enzymes, provides an ideal way to affect a single enzymic step without disturbing others. But of course, these two systems of regulation work hand in hand and provide a highly sophisticated and exquisitely sensitive overall control of cellular events.

In the early 1970's still another Ca^{2+}-dependent protein kinase, the myosin light chain kinase (MLCK) was discovered in Perry's laboratory (for a later paper see Ref. 22). MLCK, like phosphorylase kinase, the multifunctional Ca^{2+}/calmodulin-dependent protein kinase (CaM-kinase II) and others has been shown to belong to a family of enzymes dependent on Ca^{2+} and calmodulin for activity. Protein kinases responsive to second messengers other than cAMP and Ca^{2+} were eventually reported, the most notable of these being protein kinase C, which utilizes diacylglycerol as a second messenger. Indeed, the discovery of protein kinase C by Nishizuka and its relationship to phosphatidylinositol metabolism was truly one of the major events of the past decade in research on transmembrane signaling [23].

Aside from the protein kinases that are dependent on the second messengers, it was found that there are kinases whose activities are controlled by components of the systems they are called upon to regulate. Cellular functions need to be controlled during growth, differentiation, starvation, injury, etc., and cells must be able to adapt to changing environmental conditions, protect themselves against toxic or infectious agents and save energy when none is needed. As it happens, most of the switches they use to turn on or off their metabolic pathways under these conditions rely on protein phosphorylation-dephosphorylation. As an example, one can cite the control of hemoglobin synthesis which is arrested during iron or heme deficiency. Translation of globin was found to be blocked by a kinase (eIF-2 kinase) that becomes activated in the absence of heme. Likewise, during viral infection where synthesis of viral protein must be disrupted, interferon induces the synthesis of a kinase whose activity is dependent on the presence of dsRNA (for review, see Ref. 24).

Although major attention has been focused on the protein serine kinases that respond to second messengers or other effector substances (see above), it has become apparent that a great many kinases exist for which no regulatory mechanism is known, e.g., casein kinases I and II. It is possible that regulatory agents will eventually be found for some of these, or that they may regulated 'at the substrate level'. Perhaps some of these enzymes have their activities modulated by other kinases. The latter could include other protein serine kinases that are subject to regulation or certain receptor-linked protein tyrosine kinases (see below). As yet unrecognized protein–protein interactions within the cell might be involved in the regulation of independent protein kinases, e.g., by affecting their autophosphorylation.

Perhaps the most exciting development that has occurred in the protein phosphorylation field during the last 10 years relates to protein phosphorylation and cell transformation, originating from work on the Rous sarcoma virus in which the product of the *src* gene responsible for cell transformation was determined to be a protein kinase ($pp60^{v\text{-}src}$) [25]. This report was soon followed by no less surprising findings that this kinase was different from the previously known protein serine (threonine) kinases in that it phosphorylated tyrosine residues in proteins [26]. A number of other retroviral oncogenes were found to encode protein tyrosine kinases, as did their cellular protooncogenes. Then it was determined that the receptors for a family of growth factors, including EGF, insulin, IGF-1, PDGF and CSF, are protein tyrosine kinases that become activated upon the binding of the ligand [27]. Intensive work in many different laboratories is underway to determine how the phosphorylation of appropriate substrates by these receptor kinases fits into the general pattern of signal transduction. The proteins encoded by more than 20 viral oncogenes have been identified as protein kinases (predominantly protein tyrosine kinases) underlying the fundamental importance of protein phosphorylation in cell growth, differentiation and transformation [28,29]. Of course, many questions remain to be answered, most notably the identity of their substrates and the mechanism by which they induce such profound physiological changes.

With the explosion of interest in protein kinases there was a parallel development of research on protein phosphatases [30]. Indeed, for systems to be reversibly regulated, one must have enzymes that catalyze the reverse reaction, namely, the dephosphorylation of proteins. Even though studies of protein phosphatases had lagged for many years behind those on the kinases, the field has 'come into its own' lately as several of these enzymes have been purified to homogeneity and cloned. About 25 years ago, there was a suggestion on kinetic grounds that control of phosphorylase phosphorylation is exercised only by phosphorylase kinase, with no need for control of phosphorylase phosphatase. The latter enzyme was believed to have a passive role. We now know that this is incorrect and that phosphorylase

phosphatase and other phosphatases are subjected to highly complex mechanisms of regulation; furthermore, some of the phosphatases appear to be under insulin control. Finally, it has just been shown that the protein tyrosine phosphatases may represent a novel family of proteins, themselves involved in signal transduction. This followed from the finding that the two tandem cytoplasmic domains of the leukocyte common antigen (CD45) display a high degree of sequence identity with a low-molecular-weight protein tyrosine phosphatase purified to homogeneity [31] and that indeed, CD45 displays intrinsic protein tyrosine phosphatase activity [32].

In summary, it is clear that since the original observation of the interconversion of phosphorylase *b* and *a* by phosphorylation-dephosphorylation of a single serine residue on this enzyme, this type of protein modification has proven to be one of the major mechanisms by which eukaryotic cellular processes can be regulated. Protein phosphorylation has been observed in all cellular compartments and has been shown to be involved in the regulation of essentially all cellular functions. It has been estimated that almost one-third of all cellular proteins undergo phosphorylation-dephosphorylation catalyzed by perhaps one hundred or more different kinases. 35 years ago we had no idea that phosphorylase kinase would turn out to be the prototype for a family of enzymes of such critical importance and that the field would undergo such an explosive growth.

References

1 Cori, G.T. and Green, A.A. (1943) J. Biol. Chem. 151, 31–38.
2 Cori, G.T. and Cori, C.F. (1945) J. Biol. Chem. 158, 321–332.
3 Sutherland, E.W. and Cori, C.F. (1951) J. Biol. Chem. 188, 531–543.
4 Sutherland, E.W. (1951) in Phosphorous Metabolism (McElroy, W.D. and Glass, B., eds.), Vol. I, pp. 53–61, Johns Hopkins Press, Baltimore.
5 Green, A.A. and Cori, G.T. (1943) J. Biol. Chem. 151, 21–29.
6 Fischer, E.H. and Krebs, E.G. (1955) J. Biol. Chem. 216, 121–132.
7 Krebs, E.G. and Fischer, E.H. (1955) J. Biol. Chem. 216, 113–120.
8 Huston, R.B. and Krebs, E.G. (1968) Biochem. 7, 2116–2122.
9 Krebs, E.G., Kent, A.B. and Fischer, E.H. (1958) J. Biol. Chem. 231, 73–83.
10 Fischer, E.H., Graves, D.J., Snyder-Crittenden, E.R. and Krebs, E.G. (1959) J. Biol. Chem. 234, 1698–1704.
11 Sutherland, E.W. and Wosilait, W. (1955) Nature 175, 169–171.
12 Krebs, E.G., Graves, D.J. and Fischer, E.H. (1959) J. Biol. Chem. 2, 2867–2873.
13 Friedman, D.L. and Larner, J. (1963) Biochemistry 2, 669–675.
14 Linn, T.-C., Pettit, F.H. and Reed, L.J. (1969) Proc. Natl. Acad. Sci. USA 62, 234–241.
15 Walsh, D.A., Perkins, J.P. and Krebs, E.G. (1968) J. Biol. Chem. 243, 3763–3765.
16 Soderling, T.R., Hickenbottom, J.P., Reimann, E.M., Hunkeler, F.L., Walsh, D.A. and Krebs, E.G. (1970) J. Biol. Chem. 245, 6317–6328.
17 Kuo, J.F. and Greengard, P. (1969) J. Biol. Chem. 244, 3417–3419.
18 Stadtman, E.R. and Chock, P.B. (1977) Proc. Natl. Acad. Sci. USA 74, 2761–2765.
19 Krebs, E.G. and Fischer, E.H. (1962) Adv. Enzymol. 24, 263–290.
20 Cohen, P. (1982) Nature 296, 613–620.
21 Cohen, P. (1980) in Recently Discovered Systems of Enzyme Regulation by Reversible Phosphorylation (Cohen, P., ed.), pp. 255–268, Elsevier, Amsterdam.
22 Pires, E.M.V. and Perry, S.V. (1977) Biochem. J. 167, 137–146.
23 Kikkawa, U. and Nishizuka, Y. (1986) The Enzymes 17, 167–183.
24 London, I.M., Levin, D.H., Matts, R.L., Thomas, N.S.B., Petryshyn, R. and Chen, J.-J. (1987) The Enzymes 18, 360–377.
25 Collett, M.S. and Erikson, R.L. (1978) Proc. Natl. Acad. Sci. USA 75, 2021–2024.
26 Hunter, T. and Sefton, B.M. (1980) Proc. Natl. Acad. Sci. USA 77, 1311–1315.
27 White, M.F. and Kahn, C.R. (1986) The Enzymes 17, 248–302.
28 Hunter, T. and Cooper, J.A. (1986) The Enzymes 17, 192–237.
29 Hanks, S.K., Quinn, A.M. and Hunter, T. (1988) Science 241, 42–52.
30 Ballou, L.M. and Fischer, E.H. (1987) The Enzymes 17, 312–355.
31 Charbonneau, H., Tonks, N.K., Walsh, K.A. and Fischer, E.H. (1988) Proc. Natl. Acad. Sci USA 85, 7182–7186.
32 Tonks, N.K., Charbonneau, H., Diltz, C.D., Fischer, E.H. and Walsh, K.A. (1988) Biochemistry 27, 8695–8701.

Correspondence: E.H. Fischer/E.G. Krebs, Howard Hughes Medical Institute Research Laboratories, University of Washington Medical School, SL-15, Seattle, WA 98195, U.S.A.

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

by

EDWIN G. KREBS AND EDMOND H. FISCHER

Department of Biochemistry, University of Washington, Seattle, Wash. (U.S.A.)

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 3½ 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

* Supported by the initiative 171 Research Fund of the State of Washington and by a research grant (A859) from the National Institutes of Health, Public Health Service.

** 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.

References p. 157.

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*.

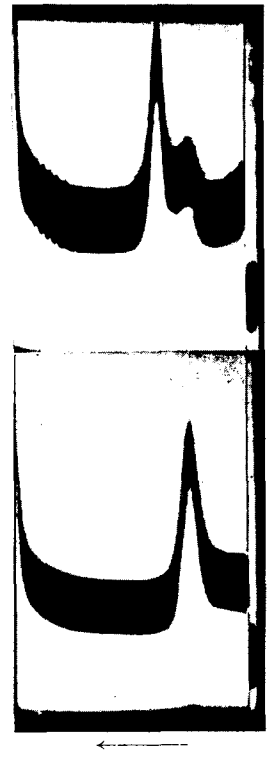

Fig. 1. Changes in the ultracentrifuge pattern during conversion of phosphorylase *b* to *a*. Centrifugations were carried out for 36 minutes in the Spinco ultracentrifuge, operating at 50,740 r.p.m., in a 0.06 *M* GP-0.03 *M* cysteine —0.1 *M* KCl buffer, pH 6.8. Upper photograph: Phosphorylase *a*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

References p. 157.

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§.

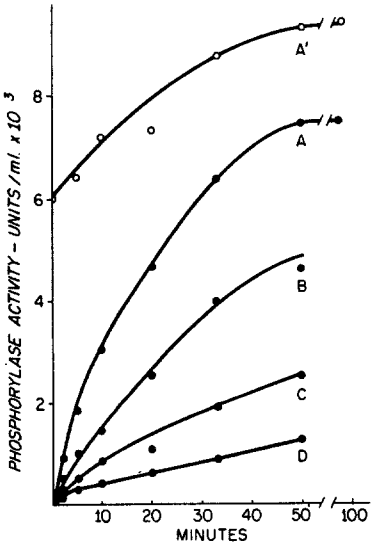

Fig. 2. Conversion of phosphorylase *b* to *a* with varying concentration of converting enzyme. Reaction mixtures were made up as described in the text for the activity test. The phosphorylase *b* solution used contained 38.0 mg protein per ml with a specific activity of 790 units/mg. Concentrations 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.

References p. 157.

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.

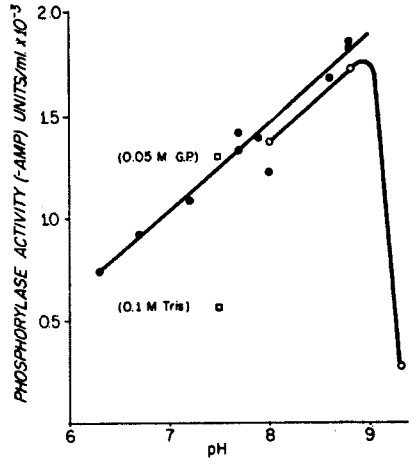

Fig. 3. Converting enzyme activity with varying pH. The composition of the reaction mixtures was the same as described in the text, except for variation in the type of buffer. Points with closed circles indicate 0.025 *M* GP–0.025 *M* Tris buffer. Points with open circles indicate 0.025 *M* GP–0.025 *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.

References p. 157.

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.

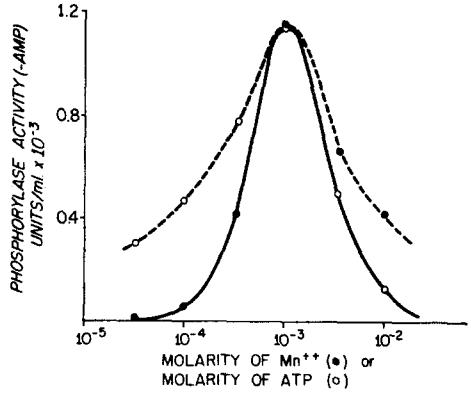

Fig. 4. Effect of variation in the ratio of Mn^{++} and ATP on conversion rates. Reaction mixture as described for the activity test, except for Mn^{++} and ATP. Converting enzyme concentration = 0.01 mg/ml. Closed circles indicate variation of $[Mn^{++}]$ with ATP constant at $10^{-3}M$. Open circles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

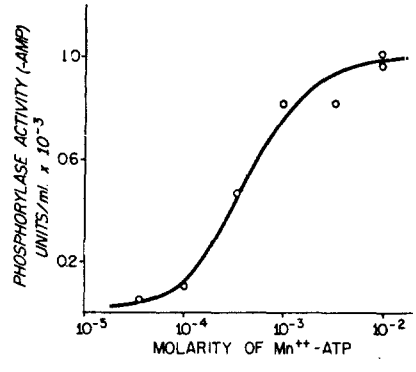

Fig. 5. Effect of variation in concentration of Mn^{++}-ATP complex on conversion rates. Reaction mixtures as in Fig. 3, except the ratio of $[Mn^{++}]$ to [ATP] constant and equal to 1. Total concentration varied.

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.

References p. 157.

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 $4.95 \cdot 10^6$ 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].

References p. 157.

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].

ACKNOWLEDGEMENTS

The authors wish to thank Mrs. JEANNE DILLS for invaluable technical assistance, Mr. BERNARDO S. VANDERHEIDEN for performing the electrophoretic analyses, and Mr. ROGER WADE for carrying out the ultracentrifuge analyses. The work performed by Mr. KENNETH ANDERSON in connection with this problem is gratefully acknowledged.

References p. 157.

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.

REFERENCES

1 G. T. CORI AND C. F. CORI, *J. Biol. Chem.*, 158 (1945) 321.
2 P. J. KELLER AND G. T. CORI, *J. Biol. Chem.*, 214 (1955) 127.
3 E. H. FISCHER AND E. G. KREBS, *J. Biol. Chem.*, 216 (1955) 121.
4 B. ILLINGWORTH AND G. T. CORI, *Biochem. Preparations*, 3 (1953) 1.
5 H. W. ROBINSON AND C. G. HOGDEN, *J. Biol. Chem.*, 135 (1940) 727.
6 A. A. GREEN, *J. Biol. Chem.*, 158 (1945) 315.
7 P. J. KELLER AND G. T. CORI, *Biochim. Biophys. Acta*, 12 (1953) 235.
8 R. D. DE MOSS, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. I, Academic Press, Inc., New York, 1955, p. 504.
9 V. A. NAJJAR, in W. D. MCELROY AND B. GLASS, *Phosphoros Metabolism*, Vol. I, Johns Hopkins Press, Baltimore, 1951, p. 500;
M. COHN, in W. D. MCELROY AND B. GLASS, *A Symposium on the Mechanism of Enzyme Action*, Johns Hopkins Press, Baltimore, 1954, p. 246.
10 G. T. CORI, B. ILLINGWORTH AND P. J. KELLER, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. I, Academic Press, Inc., New York, 1955, p. 205.
11 G. T. CORI AND A. A. GREEN, *J. Biol. Chem.*, 151 (1943) 31.
12 M. V. BUELL, *Federation Proc.*, 11 (1952) 192.
13 E. W. SUTHERLAND AND W. D. WOSILAIT, *Nature*, 175 (1955) 169.

Received November 10th, 1955