Reversibility of Phosphorylase Kinase Reaction*

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YUTAKA SHIZUTA,† RAMJI L. KHANDELWAL,$ JAMES L. MALLER,¶ JACKIE R. VANDENHEEDE,** and EDWIN G. KREBS***

From the Department of Biological Chemistry, School of Medicine, University of California, Davis, California 95616

Using a highly purified enzyme from rabbit skeletal muscle, it has been demonstrated that the glycogen phosphorylase kinase reaction is reversible. In addition to the phosphorylated protein substrate, phosphorylase a, the reverse reaction requires Mg++, ADP, Ca++, and glucose. Glucose cannot be replaced by glucose 6-phosphate or glucose 1-phosphate but can be partially replaced by glycogen. The half-maximal concentration of glucose required for the reverse reaction is approximately 25 mM at pH 8.2 and 30°. Ultracentrifugation experiments have indicated that glucose exerts its effect by facilitating the formation of a dimer from the tetrameric form of phosphorylase a, the major form present under the reaction conditions used. The quaternary structure of phosphorylase a gives a hyperbolic curve with an approximate Km value of 10 mM whereas that for phosphorylase b to a resulting in the activation of the enzyme (1), according to the following equation.

2 Phosphorylase b + 4 ATP → phosphorylase a + 4 ADP (1)

The terminal phosphate of ATP is transferred to a specific seriyl residue (2) in each subunit of phosphorylase a, which has a tetrameric structure. Heretofore, no reversal of this reaction could be demonstrated by any of several means employed (1). Studies from this laboratory to determine the mechanism of action of adenosine 3′:5′-monophosphate in stimulating glycogenolysis revealed that phosphorylase kinase in skeletal muscle is activated by phosphorylation catalyzed by a separate protein kinase, an enzyme that was designated as the cyclic AMP-dependent protein kinase (3–5). The properties of this latter enzyme as well as those of phosphorylase kinase have been discussed in recent reviews (6–8). The methods for obtaining homogeneous preparations of both kinases from rabbit skeletal muscle have also been described (9, 10).

It has been demonstrated that several protein kinase reactions are reversible. Rabinowitz and Lipmann (11) first reported that phosphotran kinase, which was partially purified from yeast or brain, catalyzed a reversible reaction when phosphorylated phosphotran was used as the substrate. Lerch et al. (12) purified a similar enzyme to homogeneity from baker's yeast and also observed that it catalyzed a reversible reaction using casein or phosphotran as the substrate. Shizuta et al. (13) demonstrated that homogeneous rabbit muscle cyclic AMP-dependent protein kinase catalyzed a reversible phosphorylation reaction when any of several protein substrates were used. Rosen and Erlichman (14) showed a reversible transfer of phosphate in the autophosphorylation reaction of bovine heart cyclic AMP-dependent protein kinase.

In the present study, it was found that the phosphorylase kinase reaction, like the other protein kinase reactions described above, is also reversible. The probable reason for the discrepancy between the present finding and that previously reported (1) lies in the fact that only the dimeric form of phosphorylase a will serve in this process.

EXPERIMENTAL PROCEDURES

Materials

Nonactivated phosphorylase kinase was prepared according to the method of Hayakawa et al. (10) except that the buffer solution was supplemented with 15 mM β-mercaptoethanol in the Sepharose 4B

† The abbreviations used are: cyclic AMP, adenosine 3′:5′-monophosphate; TGF buffer, 0.125 M Tris, 0.125 M β-glycerophosphate buffer, pH 8.5.

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**To whom reprint requests should be addressed.
assay of the reverse reaction. Unless indicated otherwise nonactivated phosphorylase kinase was used in all of the experiments reported in this paper. The activated form of phosphorylase kinase was prepared by preincubating it in the presence of MgATP and the catalytic subunit of the cyclic AMP-dependent protein kinase as described by Hayakawa et al. (15). Crystalline phosphorylase b was obtained by the method of Fischer and Krebs (16). AMP in the crystalline enzyme was removed by charcoal treatment. Crystalline [32P]phosphorylase a was prepared by the following modification of the method of Krebs and Fischer (17). The standard reaction mixture in a final volume of 6.7 ml contained 1.0 ml of Tris/glycophosphate (TPG) buffer (pH 8.6), 215 pmol of AMP-free phosphorylase b, 5 pmol of [γ-32P]ATP (1,000 cpm/pmol), 50 pmol of magnesium acetate, 5 nmol of magnesium chloride, 10 μmol of glucose, and 0.1 mg of phosphorylase kinase. Incubation was performed at 30° for 30 min. The tube containing the reaction mixture was then cooled in ice for 30 min. The crystals formed were collected by centrifugation at 4° and dissolved in 2 ml of TGP buffer (pH 8.6) containing 50 mm β-mercaptoethanol prewarmed to 30°. Recrystallization was performed three times by the above procedure. The sample solution was then treated twice with 50 mg of charcoal at room temperature for 15 min. The charcoal was removed by centrifugation at 20°. The protein solution was dialyzed overnight at 4° against 1 liter of TGP buffer (pH 8.6) containing 10 mm β-mercaptoethanol. The enzyme crystallized during dialysis. The [32P]phosphorylase a crystals thus obtained contained 0.94 pmol of 32P per monomer subunit, assuming the molecular weight of the monomer to be 90,000 (18). The ratio of the optical density at 260 nm to that at 280 nm was 0.55. All other materials including enzymes and chemicals were obtained as described previously (19).

Methods

Assay of Phosphorylase Kinase Activity—Enzyme activity for the forward reaction was determined by measuring the incorporation of 32P into phosphorylase b using the filter paper method described by Reimann et al. (19). The standard reaction mixture contained 20 μl of TGP buffer (pH 8.6), 0.5 mg of phosphorylase b, 10 nmol of γ-32P ATP (specific activity 400 to 600 cpm/pmol), 1 μmol of magnesium acetate, 10 nmol of calcium chloride, 10 μmol of glucose, and varying amounts of phosphorylase kinase in a total volume of 0.1 ml. The final pH of the reaction mixture was 8.2. Incubation was performed at 30° for 2 min.

The activity of the kinase for the reverse reaction was determined by the following procedure. The standard reaction mixture contained 20 μl of TGP buffer (pH 8.6), 0.6 mg of [32P]phosphorylase a as described above, 10 μmol of glucose, 1 μmol of ADP, and varying amounts of phosphorylase kinase in a total amount of 0.1 ml. The final pH was 8.2. The control reaction mixture contained the same reaction components except for the omission of ADP. Incubation was performed at 30° for 15 min. The reaction was terminated by adding 0.1 ml of cold 20% trichloroacetic acid and the mixture was kept at 4° for 15 min. The precipitate was removed by centrifugation and an aliquot of the supernatant solution, containing the [γ-32P] ATP formed, was taken for the determination of radioactivity.

Identification of Nucleotide Reaction Product of Reverse Reaction as γ-32P ATP—The reaction mixture contained 5 μmol of calcium chloride, 2 μmol of ADP, 1.44 mg of [32P]phosphorylase a, 20 μmol of glucose, and 0.1 mg of phosphorylase kinase in a total volume of 0.2 ml. Incubation was performed at 30° for 60 min. The reaction was stopped by the addition of 0.2 ml of 20% trichloroacetic acid and the precipitate was removed by centrifugation. The supernatant solution was added 50 mg of charcoal and the mixture was shaken vigorously for 5 min on a Vortex mixer. The subsequent washing and elution procedures were the same as those described previously (13). More than 90% of the radioactive compound released from [32P]phosphorylase a into the trichloroacetic acid supernatant was adsorbed by the charcoal and the overall recovery was approximately 60%. The radioactive product thus isolated was shown to be identical with γ-32P ATP using each of the methods described previously (13).

Ultracentrifugal Analyses—Sedimentation velocity experiments were performed in a Beckman model E ultracentrifuge equipped with schlieren optics. The rotor temperature was set at 30°. The data were corrected to water at 20°.

Other Analytical Methods—The protein concentrations of phosphorylase a and b were determined using an ε2.8 value of 11.9 (20). The concentrations of phosphorylase kinase were determined using
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The complete reaction mixture contained 20 μl of TGP buffer (pH 8.6), 1 μmol of magnesium acetate, 0.05 μmol of calcium chloride, 10 μmol of glucose, 0.01 μmol of ethylene glycol bis(β-aminoethyl ether)N,N'-tetraacetic acid, 1 μmol of ADP, 0.16 mg of phosphorylase a containing 10,260 pmol of 32P (specific activity 942 cpm/pmol), and 20 μg of phosphorylase kinase in a total volume of 0.1 ml. After incubation at 30°C for 15 min, radioactivity in trichloroacetic acid supernatants was determined by the same procedure as described in the legend of Fig. 1.

| Components | 32P liberated (cpm) |
|------------|---------------------|
| Complete system | 17,400 |
| Glucose | 640 |
| ADP | 150 |
| Ca2+ | 150 |
| Mg2+ | 40 |
| Enzyme | 0 |

**Table II**

Effects of various compounds on reverse reaction of phosphorylation kinase

The reaction mixtures in a final volume of 0.1 ml contained 20 μl of TGP buffer (pH 8.6), 1 μmol of magnesium acetate, 0.01 μmol of calcium chloride, 1 μmol of ADP, 0.18 mg of [32P]phosphorylase a containing 1400 pmol of 32P (specific activity 347 cpm/pmol), and 30 μg of phosphorylase kinase in the absence and presence of various additions as indicated. The control reaction mixtures contained the same components except for the omission of ADP. Liberation of 32P from [32P]phosphorylase a was determined by the same procedure as described in the legend for Fig. 1. Results are expressed as the ADP-dependent liberation of 32P from [32P]phosphorylase a, i.e. the small amount of trichloroacetic acid-soluble radioactivity in the control experiments without ADP has been subtracted.

| Additions | 32P liberated | Per cent stimulation by ligand |
|-----------|-------------|-----------------------------|
| None | 1,500 | - |
| 2.8 M NaCl | 1,540 | 3 |
| 1% glycerol | 8,100 | 440 |
| 0.1 M glucose | 36,000 | 2,500 |
| 0.1 M glucose | 36,000 | 2,500 |
| 10 mM glucose-6-P | 2,100 | 40 |
| 10 mM glucose-1-P | 1,500 | 0 |
| 10 mM AMP | 1,200 | -20 |
| 10 mM Pi | 1,400 | -7 |

an ATP value of 11.8 (10). The concentrations of other proteins were determined by the method of Lowry et al. (21). All other analytical methods were performed as described previously (13).

**RESULTS**

Glucose-dependent Phosphate Transfer from [32P]Phosphorylase a to ADP—As noted above, an early attempt to demonstrate the occurrence of a reverse reaction catalyzed by phosphorylase kinase was unsuccessful (1). In the present study, however, it was noted that when reaction mixtures were supplemented with a relatively high concentration of glucose, liberation of radioactivity from [32P]phosphorylase a into a trichloroacetic acid-soluble form occurred in the presence of ADP (Fig. 1). This reaction proceeded linearly for 30 min. On the other hand, almost no radioactivity was released in the absence of ADP. The radioactive reaction product liberated into the trichloroacetic acid-soluble fraction was isolated by adsorption on charcoal and identified as γ-[γ-32P]ATP as described under "Methods." More than 90% of the reaction product was adsorbed by the charcoal and there was no indication to suggest that glucose was directly involved in the reaction, i.e. that any sugar/phosphate compound was being formed. These findings indicate that the phosphorylase kinase reaction is reversible under these conditions.

In the experiment shown in Fig. 2, the effect of phosphorylase kinase concentration on the liberation of radioactivity from [32P]phosphorylase was examined in the presence and absence of ADP. As indicated in this figure, the amount of radioactivity liberated from [32P]phosphorylase a in the presence of ADP (i.e. the amount of γ-[γ-32P]ATP formed) was linear up to an enzyme concentration of 200 μg/ml.

Another set of experiments was performed to see what components were required for the reverse reaction. The results are summarized in Table I. In addition to ADP, Ca2+ and Mg2+ were essential for the glucose-dependent reaction, consistent with the known requirements of the enzyme in catalysis of the forward reaction (3, 22).

Characteristics of Glucose Effect It was found that the glucose effect in the reverse reaction became less marked when an aged preparation of phosphorylase a was employed as the substrate. Namely, in this case the reverse reaction proceeded to a considerable degree without glucose. Furthermore, procedures that might modify the conformation of phosphorylase a, such as prolonged dialysis against TGP buffer at 30-37°C or freezing and thawing, facilitated the reverse reaction in the absence of glucose. These observations, together with the reports by other investigators (23, 24), suggested that glucose exerts its effect by modifying the conformation of the protein substrate, phosphorylase a. The effects of various substances, including other ligands, on the reverse reaction of phosphorylase kinase were then examined using a fresh crystalline preparation of phosphorylase a. Table II shows the results of a typical experiment. It is seen that glucose was most effective and glycogen was partially effective in promoting the reverse reaction. Glucose could not be replaced by other compounds.
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FIG. 4. Effect of glucose on sedimentation patterns of phosphorylase a and phosphorylase kinase. The sample materials contained 25 mM Tris, 25 mM β-glycerophosphate, 10 mM magnesium acetate, 0.1 mM calcium chloride, 10 mM ADP, and 6 mg/ml of phosphorylase a or 1.5 mg/ml of phosphorylase kinase in the presence (top) and absence (bottom) of 0.1 mM glucose. The final pH was 8.2. Ultracentrifugation was performed at 30°C and 52,000 r.p.m. Sedimentation is from right to left.

such as glucose 1-phosphate or glucose 6-phosphate under the conditions employed, although there is a slight suggestion that the latter compound had some effect.

In the experiments shown in Fig. 3, the effect of glucose concentration on the reverse reaction of the kinase was examined using two different concentrations of phosphorylase a. The results indicate that the concentration of glucose giving a half-maximal effect is approximately 25 mM whereas 80 to 200 mM glucose is required for the maximal effect.

It was determined that at a concentration of glucose causing a maximal increase in velocity of the reverse reaction, i.e. 0.1 M, phosphorylase a is apparently completely dissociated from its tetrameric to its dimeric form (Fig. 4). Thus, in the absence of glucose, the enzyme sedimented as a single somewhat asymmetric peak with an s20,w value of 13.1 whereas in the presence of 0.1 M glucose the s20,w value was 8.6. The symmetry of the peak was noticeably greater under the latter condition. These sedimentation values correspond closely to those reported for the tetrameric and dimeric forms of phosphorylase respectively. On the other hand, glucose did not induce a marked change in the s20,w value of phosphorylase kinase.3

The effects of elevated temperature and high salt on the reverse reaction and on the quaternary structure of phosphorylase a were also examined, since it has been reported that these conditions facilitate the dissociation of phosphorylase a into its dimeric form (25–27) as do glucose (23, 24) and several other carbohydrates including glycogen (28–30). Neither conditions stimulated the reverse reaction. It is possible, however, that the high salt concentration (see Table III) may have caused almost complete inhibition of the enzyme even in the forward reaction (3). It is also possible that elevation of the temperature (to 37°C) did not cause appreciable dimer formation in the reaction mixture due to the presence of 10 mM ADP.

FIG. 5. Effect of pH on the forward (Ο) and the reverse (●) reactions of phosphorylase kinase. Each reaction velocity was determined using the standard assay conditions except for changing the pH of the buffer solution.

Characterization of Glucose-dependent Reverse Reaction of Phosphorylase Kinase—Fig. 5 shows the pH profile for the forward and the reverse reactions of phosphorylase kinase. As shown in this figure, the optimum pH for the forward reaction was approximately 9. The ratio of activity at pH 6.8 to activity at 8.2 was 0.06, as is characteristic for nonactivated (nonphosphorylated) phosphorylase kinase (4). When the enzyme was activated by preincubation with MgATP in the presence of the catalytic subunit of cyclic AMP-dependent protein kinase (see "Experimental Procedures"), the pH 6.8 to pH 8.2 activity ratio increased to 0.5 for the forward reaction as anticipated (3, 4, 15). In contrast, the optimum pH of the reverse reaction was in the range of 6.8 to 7.6 before and after the enzyme was activated by the cyclic AMP-dependent protein kinase catalytic subunit. (The data for the activated form of the kinase are not illustrated.)

As shown in Fig. 6, the reverse reaction velocity as a function of [32P]phosphorylase a gave a hyperbolic curve with an approximate Kₘ value of 40 mg/ml. On the other hand, the reverse reaction velocity plotted against ADP concentration using three different concentrations of phosphorylase a gave biphasic curves (Fig. 7).

Table III shows the results of a typical experiment in which the nucleotide substrate specificity of the enzyme for the reverse reaction was examined. The best nucleotide substrate was ADP, but GDP also served as a substrate for the reverse reaction. Other nucleotides did not serve effectively as acceptors of phosphate.

DISCUSSION

The present study has shown that phosphorylase kinase can catalyze the reversal of the phosphorylase b to phosphorylase a reaction under certain conditions. This conclusion is based on
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FIG. 6 (left). Effect of phosphorylase $a$ concentration on the reverse reaction. Reaction velocities were determined using the standard assay conditions for the reverse reaction as described under "Methods" except for using varied concentrations of $[^{32}P]$phosphorylase $a$.

FIG. 7 (right). Effect of ADP concentration on the reverse reaction. Reaction velocities were measured under the standard assay condition for the reverse reaction except for using varied concentrations of ADP.

### Table III

Specificity of nucleotide substrate for reverse reaction

| Nucleotide substrate | $^{32}P$ transferred pmol/min/mg |
|----------------------|---------------------------------|
| ADP                  | 408                             |
| GDP                  | 256                             |
| GTP                  | 0                               |
| UDP                  | 0                               |
| TDP                  | 93                              |
| IDP                  | 47                              |
| XDP                  | 3                               |
| AMP                  | 0                               |

It is important to note that the dimeric form of phosphorylase $a$ is a better substrate than the tetrameric form in the phosphonphatase reaction (25, 26). It is also noteworthy that the dimeric form of phosphorylase $a$ is much more active than the tetrameric form of the same protein with respect to its glycogenolytic activity (23, 28, 30). The authors recognize that properties attributed to dimeric phosphorylase $a$, in contrast to those of the tetrameric form, may in reality be due to a particular conformational form induced by an effector such as glucose. Such a form may coincidentally have less ability to polymerize to the tetrameric form.

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