Interactions between Native and Chemically Modified Subunits of Matrix-Bound Glycogen Phosphorylase*

(hybrid enzyme/monomers/dimers/pyridoxal-phosphate)

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ABSTRACT Phospho-dephosphohybrids of rabbit skeletal muscle phosphorylase (EC 2.4.1.1; α -1,4-glucan: orthophosphate glucosyl transferase) have been prepared and stabilized by attachment to Sepharose activated by cyanogen bromide. They can be distinguished from phosphorylase *a* by their sensitivity to inhibition by glucose-6-phosphate and activation by adenosine 5'-monophosphate. Stable hybrids have also been formed between phosphorylase subunits containing the active cofactor pyridoxal-phosphate and inactive analogs (pyridoxal-phosphate monomethylester or the corresponding reduced compounds). After complete dissociation to monomers, the Sepharose-bound phosphorylase had a residual activity of less than 3% of that of the original matrix-bound dimeric enzyme. The hybrid enzyme is composed of a potentially active subunit containing pyridoxal-phosphate and an intrinsically inactive subunit carrying the analog, and it had half the activity of the original dimeric enzyme. Thus, the interaction of the inactive subunit with matrix-bound phosphorylase monomers elicited activity in the monomers.

Fischer *et al.* (1,2) observed, in the course of *in vitro* interconversion of phosphorylase *b* $\rightleftharpoons$ *a*, catalyzed by phosphorylase kinase (EC 2.7.1.38) and phosphorylase phosphatase (EC 3.1.3.17), an enzyme that was much more sensitive to inhibition by glucose-6-P than the fully phosphorylated enzyme and that was, therefore, assumed to be a phospho-dephosphohybrid. However, all attempts to isolate the phospho-dephosphohybrids failed, presumably because the soluble hybrid molecules rearranged to form fully phosphorylated and nonphosphorylated oligomers. Interest in the physiological role of such hybrids was greatly stimulated by the recent finding (3) that the "flash activation" of phosphorylase bound to the glycogen "organelle" of rabbit muscle upon addition of Ca^{++} , ATP, and Mg^{++} produced an enzyme species that was about 40% inhibited by glucose-6-P. This suggested the formation of phospho-dephosphohybrids in intact muscle during phosphorylase activation in response to muscle contraction. The stabilization and the properties of phospho-dephosphohybrids bound to Sepharose are described here.

Meighen and Schachman (4) have hybridized native and succinylated subunits of muscle aldolase and glyceraldehyde-

3-P dehydrogenase. They concluded that each subunit expressed its activity independently of the other. Activity can be induced in inactive phosphorylase monomers by hybridization. In the hybrid phosphorylase, the subunit expressed its individual activity.

MATERIALS AND METHODS

Phosphorylase *b* was prepared from fresh rabbit skeletal muscle and recrystallized at least three times before use (5). AMP was removed by passage over charcoal ($A_{260}:A_{280} \leq 0.53$) (6). Pyridoxal-P was resolved from the protein by the procedure of Shaltiel *et al.* (7). Apophosphorylase *b* (50 μM monomer) was reconstituted with 60 μM pyridoxal-P, pyridoxal-P ester, or 0.5 mM pyridoxal. All calculations are based on a molecular weight of 100,000 for the phosphorylase monomer that contains one specific binding site for pyridoxal-P (8). Native or reconstituted phosphorylase *b* was reduced with NaBH_4 (9). Soluble or Sepharose-bound phosphorylase *b* was converted to the *a* form with phosphorylase *b* kinase, ATP, and Mg^{++} (10). Phosphorylase *a* was converted to *b* with phosphorylase phosphatase prepared from the 80,000 $\times g$ pellet obtained in the preparation of the glycogen-phosphorylase organelle (11, 12). Protein concentrations were determined at $A_{280}^{1\% \text{ cm}^{-1}} = 13.2$ (6) or by the Lowry method (13). Specific activities ($\mu\text{mol of P}_i \times \text{mg}^{-1} \times \text{min}^{-1}$) of freshly prepared phosphorylase ranged from 70 to 75 with 1 mM AMP for phosphorylase *b*, and from 57 to 60 for phosphorylase *a* without AMP, and from 63 to 68 with AMP. Sepharose 4B was carefully washed with water to remove NaN_3 . 5-ml Batches of packed Sepharose diluted with an equal volume of water were usually reacted with 10 mg of CNBr at 20° for 8-10 min (14, 15). The pH was kept at 11 by addition of 1 N NaOH. The reaction mixture was rapidly cooled with ice, filtered, and washed with 150 ml of ice-cold 50 mM glycerol-P buffer (pH 7.0) in less than 1 min. Activated Sepharose was rapidly added to 5 ml of a solution containing phosphorylase (2-15 mg/ml) in 50 mM glycerol-P buffer (pH 7.0). The mixture was gently stirred for 3 hr in the cold and left at 4° overnight. Sepharose-enzyme was washed five times with 25 ml each time of 50 mM glycerol-P-30 mM L-cysteine buffer (pH 7.0) and left at 4° for 12 hr. The remaining soluble protein was finally removed with 50 mM glycerol-P-50 mM 2-mercaptoethanol buffer (pH 7.0). 0.1-0.4 ml of Sepharose-bound enzyme was placed into a small Plexiglass column (3.8-mm diameter), jacketed for temperature control. The lower end of the column was closed with a perlon-diaphragm (no. 3803, Pharmacia). The substrate mixture [100 mM glucose-1-P-1% glycogen with or without 1 mM AMP in 25 mM 2-mercaptoethanol-100 mM glycerol-P buffer (pH 6.8)] was pumped through the column

Abbreviations: ClHgBz, *p*-chloromercuribenzoate; SDS, sodium dodecyl sulfate.

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at a constant flow rate with a Perplex pump (model A 10, 200 LKB). The amount of enzyme and the flow rate were adjusted so as not to exceed a product (P_i) concentration of 1.2 mol/200 liter. After about 2.5 min, a steady state was reached. 200- μ l samples were then removed and analyzed for P_i (16). If the drive gear was changed or tubing of different diameter was inserted, the flow rate and the time the substrate was in contact with the enzyme changed accordingly. The time required for a given volume (Δv) to pass through the column is inversely proportional to the flow rate ($f = dv/dt$). A plot of the amount of product ($\mu\text{mol } P_i/\Delta v$) against the time (Δt) this aliquot needs to pass through the column yields the rate of the enzymatic reaction. The linear part is shown in Fig. 1A. The apparent K_m or K_a values of matrix-bound phosphorylase for substrate and activator are easily determined (Fig. 1B, Table 1). The concentration of reduced phosphorylase in the column was determined by tritiation with $[^3\text{H}]\text{KBH}_4$ (290 Ci/mol). The tritiated enzyme was exhaustively dialyzed to remove exchangeable tritium. The protein-bound radioactivity was only about 20% of that of the specific radioactivity of $[^3\text{H}]\text{KBH}_4$. Aside from the removal of exchangeable tritium, this is a consequence of a primary isotope effect. Native phosphorylase *b* was labeled with $[^{14}\text{C}]\text{iodoacetamide}$ (5 Ci/mol) (17). Usually about 4 SH groups per dimer *b* were blocked (1 mg of protein contained about 2.2×10^4 dpm). The enzymatic activity of the carboxyamidomethylated enzyme was even higher than that of the unreacted phosphorylase (18) ($75\text{--}80 \mu\text{mol } P_i \times \text{min}^{-1} \times \text{mg}^{-1}$). Radioactivity was measured in the Sepharose-bound enzyme by transfer of a measured amount of the contents of the column into a counting vial and hydrolysis with 0.5 ml of 12 N HCl. 0.5 ml of water and 15 ml of a solution containing 7.28 g of 2,5-diphenyloxazol and 0.72 g of *p*-bis-(*o*-methylstyryl)benzene per liter of a 2:1 mixture of toluene and Triton X-100 were added. The white, lumpy precipitate was completely dissolved, and the samples were counted in a Packard liquid scintillation spectrometer. Dimers *b* and *a* of the phosphorylase bound to the matrix were dissociated to monomers by treatment with 0.8 M imidazole citrate buffer (pH 6.2) (without L-cysteine which would remove pyridoxal-P!). Soluble phosphorylase *b*

TABLE 1. Kinetic properties of soluble and Sepharose-bound phosphorylase

Phosphorylase preparations	Specific activity		K_m [Glucose-1-P]		K_a [AMP]
	-AMP	+1 mM AMP	-AMP	+1 mM AMP	
	$(\mu\text{mol } P_i \cdot \text{mg}^{-1} \cdot \text{min}^{-1})$		(mM)		
Soluble dimer <i>b</i>	2.5	75	—	2.2–5.5*	10–70*†
Sepharose-bound dimer <i>b</i>	0.5–1	13–25	—	5.8	20
Soluble dimer <i>a</i>	57‡	65‡	5.3‡	1.8‡	1–2‡
Sepharose-bound dimer <i>a</i>	16	20	5.9	4.4	—
Sepharose-bound hybrid <i>b-a</i>	8	23	—	4.9	2–4

* According to (6); † according to (28), ‡ according to (29).

FIG. 1. (A) Activity measurements of matrix-bound phosphorylase dimer *b*. Tube diameter about 1.1 mm, gear box transmission ratios: 3:250, $\circ$; 9:500, $\square$; 3:125, ∇ ; tube diameter about 1.3 mm, ratios 3:250, $\bullet$; 9:500, $\blacksquare$, 3:125, $\blacktriangledown$. (B) Apparent K_m of glucose-1-P and matrix-bound dimer *a*. Initial velocities were measured in the presence of 1 mM AMP. The temperature was 30° .

and *a* (1 mg/ml), and in some cases, matrix-bound phosphorylase *a* were dissociated by treatment with 0.2 mM ClHgBz (19). In some experiments (see Fig. 2), Sepharose-bound dimer *b* was dissociated with 0.2–2% sodium dodecyl sulfate (SDS). Extent of dissociation of the soluble dimeric enzyme at the concentrations used in the hybridization experiments (0.5 mg/ml) was checked at 20° in the analytical ultracentrifuge equipped with a UV-light scanner. The slowly sedimenting species had an *s* value of 5.8 (19). The dissociated subunits were washed from the column with

FIG. 2. Dissociation of matrix-bound phosphorylase dimer *b*. The experiments were performed at 30° .

TABLE 2. *Matrix-bound phospho-dephosphohybrids*

Experiments	Sephacrose-bound phosphorylase	Activity	
		– AMP	+1 mM AMP
		$(\mu\text{mol Pi} \cdot \text{min}^{-1})$	
1	Dimer <i>a</i>	0.61	0.71
	Monomer <i>a</i>	0.046	0.063
	Hybrid ($a^B \leftrightarrow b^S$)	0.29	0.62
	Dimer <i>a</i> (obtained from hybrid by kinase)	0.48	0.63
2	Dimer <i>b</i>	0.02	0.69
	Monomer <i>b</i>	0	0.07
	Hybrid ($b^B \leftrightarrow a^S$)	0.27	0.63
	Hybrid ($b^B \leftrightarrow a^S$) (with 5 mM glucose-6-P)	0.14	0.57
	Dimer <i>b</i> (obtained from hybrid by phosphatase)	0.02	0.63
3	Dimer <i>b</i>	0.02	0.60
	Monomer <i>b</i>	0	0.057
	Hybrid ($b^B \leftarrow$ pyridoxal-P-ester a^S)	0.04	0.29

Each of the experiments was repeated at least twice. Variations in activity of matrix-bound dimers in different experiments result from different enzyme concentrations.

B is the subunit bound covalently to Sepharose and *S* is the soluble subunit. The direction of induction is indicated by the arrow.

imidazole-citrate buffer. Finally, the column was washed with 50 mM glycerol-P-50 mM 2-mercaptoethanol 10 μ M pyridoxal-P buffer (pH 7.0). Soluble phosphorylase monomers were then added to the Sepharose-bound monomers, and hybridization was initiated by removal of ClHgBz with 100 mM 2-mercaptoethanol-50 mM glycerol-P buffer (pH 6.8) at 30°. After 1–2 hr, the column was again carefully washed for at least 1 hr until all soluble material was removed.

Oyster glycogen, pyridoxal-P, pyridoxal, and imidazole were products of E. Merck. Pyridoxal-P ester was prepared

TABLE 3. *Interaction between a monomer containing active and inactive pyridoxal-P analogs*

Experiments	Preparations	Activity	
		– AMP	+1 mM AMP
		$(\mu\text{mol Pi} \cdot \text{min}^{-1})$	
1	Dimer <i>a</i> (PLP ^B ·PLP ^S)	0.63	0.80
	Monomer <i>a</i> (PLP ^B)	0.05	—
2a	Dimer <i>a</i> (PLP ^B $\leftrightarrow$ PMP ^S)	0.50	0.60
	Dimer <i>a</i> (PLP ^B ·PLP ^S)	0.63	0.79
	Monomer <i>a</i> (PLP ^B)	0.06	—
2b	Hybrid dimer <i>a</i> (PLP ^B $\leftarrow$ PLP-ester ^S)	0.33	0.42
	Dimer <i>a</i> (PLP ^B ·PLP ^S)	0.59	0.73
	Monomer <i>a</i> (PLP ^B)	0.07	—
	Hybrid dimer <i>a</i> (PLP ^B $\leftarrow$ PMP-ester ^S)	0.24	0.33

See legend to Table 2. PLP, pyridoxal phosphate; PMP, pyridoxamine phosphate; other abbreviations are as in Table 2.

according to (18). Nucleotides and sugar phosphates were obtained from Boehringer and Sons. Sepharose 4B was purchased from Pharmacia, Uppsala; 2-mercaptoethanol, protamine sulfate, bovine-serum albumin, SDS, and ClHgBz were purchased from Serva, Heidelberg; [¹⁴C]iodoacetamide and [³H]KBH₄ were purchased from the Radiochemical Centre, Amersham, England. The scintillators were obtained from Zinsser, Frankfurt, and Triton X-100 was obtained from Rohm and Haas.

RESULTS

Properties of Sepharose-bound phosphorylase (Table 1)

There was little difference between apparent *K_m* and *K_a* values of glucose-1-P and AMP for soluble and Sepharose-bound phosphorylases. Matrix-bound phosphorylase *b*, however, exhibited no homotropic cooperativity with respect to AMP activation. The specific activities of matrix-bound phosphorylase *b* or *a* were 15–33% of the original enzyme, depending on the extent of activation of Sepharose by CNBr.

Based on the activity of matrix-bound phosphorylase dimer *b* (100%), a small residual activity (<10%) remained after dissociation with imidazole citrate. This posed the question of whether the residual activity represented the basal activity of monomeric phosphorylase. For each point on the

TABLE 4. *Specificity of subunit interactions*

Experiments	Preparations	Activity	
		– AMP	+1 mM AMP
		$\mu\text{mol Pi} \cdot \text{min}^{-1}$	
1	Dimer <i>a</i> (PLP ^B ·PLP ^S)	0.60	0.74 $\pm$ 0.04
	Monomer <i>a</i> (PLP ^B)	0.059 $\pm$ 0.012	—
	Hybrid dimer <i>a</i>	0.19 $\pm$ 0.05	0.25 $\pm$ 0.06
	(PLP ^B $\leftarrow$ PM ^S)	(0.30)*	(0.37)*
2	Dimer <i>b</i> (PLP ^B ·PLP ^S)	0.02	0.60
	Apo- <i>b</i> monomer	—	0.005 $\pm$ 0.003
	Hybrid dimer <i>b</i> (Apo ^B $\rightarrow$ PMP ^S)	—	0.063 $\pm$ 0.004
	Reconstituted dimer <i>b</i> (PLP ^B $\leftrightarrow$ PMP ^S)	—	0.14 $\pm$ 0.01
3a	Dimer <i>a</i> (PLP ^B ·PLP ^S)	0.60	0.70
	Monomer <i>a</i> (PLP ^B)	0.05	—
	Monomer <i>a</i> with protamine sulfate†	0.05	0.06
	with serum albumin†	0.05	0.06
3b	Dimer <i>b</i> (PLP ^B ·PLP ^S)	0.02	0.65
	Monomer <i>b</i> (PLP ^B)	0	0.063
	Monomer <i>b</i> with protamine†	0	0.065
	sulfate with serum albumin†	0	0.068

See legend to Table 2. Experiments 1 and 2 were each repeated five times. Pyridoxal-P was resolved from matrix-bound phosphorylase *b*, and reconstitution of the matrix-bound apophosphorylase *b* with pyridoxal-P was performed as described for the soluble phosphorylase proteins (7).

* Theoretically expected activity on complete induction is given in parentheses.

† The concentration of bovine serum albumin was 1 mg/ml and the concentration of protamine sulfate was 20 μ g/ml. Abbreviations are as in Tables 2 and 3; PM, pyridoxamine.

graph (Fig. 2), residual enzymatic activity and protein concentration of the column were determined after partial dissociation. The starting concentration (100%) was determined for each point from the sum of the radioactivity remaining in the column (ordinate) and the radioactivity in the eluate. When the covalently bound monomer is as active as the dimer, complete monomerization would result in the loss of 50% of the enzymatic activity of the dimer bound to the column ($\tan \alpha = 1$). Conversely, if the Sepharose-bound monomers are inactive, removal of 50% of the radioactivity of the column would indicate dissociation to monomers and should result in complete loss of activity ($\tan \alpha = 1/2$). The curve obtained demonstrates, however, that dissociation of Sepharose-bound phosphorylase *b* after treatment with imidazole citrate was neither uniform nor complete. About 68% of the radioactivity remained bound. The excess (18%) over the covalently bound monomers (50%) could be reduced to less than 1% by treatment with SDS in glycerol-P buffer (pH 7.0). Thus, the residual matrix-bound enzymatic activity represents most likely dimeric phosphorylase with low specific activity that was resistant to dissociation after treatment with imidazole citrate. Phosphorylase was initially present in the column nearly exclusively as dimers covalently linked to the matrix by only one subunit. SDS, which dissociated Sepharose-bound phosphorylase *b* more effectively, forms an inactive complex with the enzyme. After resolving the detergent from the enzyme with serum albumin and reconstitution with pyridoxal-P, the remaining monomeric activity was 1%. On dimerization with soluble phosphorylase subunits, 30% of the starting activity was regained. Matrix-bound phosphorylase monomer *b* has, therefore, 3% of the activity of the Sepharose-bound dimer *b*.†

Phospho-dephosphohybrids

In experiment 1 in Table 2, soluble *b* monomers were added to matrix-bound *a* monomers. Clearly, the phospho-dephosphohybrid is much more dependent on AMP for activity than phosphorylase *a*. The gain in activity with AMP is about 110% for the hybrid but 20% for phosphorylase *a*. Moreover, on addition of soluble subunits to matrix-bound monomers, activity in the presence or absence of AMP was much greater than that of the matrix-bound monomers (see also experiment 2). The amount of AMP required for half maximal activation of the *b*-*a* hybrid was considerably less (2-4 μ M) than that required for activation of phosphorylase *b* (20 μ M) (Table 1). In this respect, the hybrid resembled phosphorylase *a*. This suggests that the *b*-*a* hybrid has different control properties with respect to AMP activation than phosphorylase *b* (also, see 1, 20). Phosphorylation of the hybrid with ATP-Mg⁺⁺, catalyzed by phosphorylase *b* kinase, resulted in an increase exclusively of AMP-independent activity indicating conversion to phosphorylase *a* (experiment 1).

The reverse experiment with matrix-bound *b* monomers and soluble *a* monomers confirmed the results. The interaction between bound *b* monomers and soluble *a* monomers induced

AMP-independent activity, which again disappeared on complete dephosphorylation (experiment 2). This experiment was repeated with soluble *a* monomers containing ³²P introduced by the phosphorylase *b* kinase reaction from [γ -³²P]-ATP. The results gave additional proof for the formation of a hybrid *b*-*a* dimer, because the ³²P-labeled *a* monomer could only be removed from the matrix-bound *b* subunit with imidazole citrate. Moreover, the extent of dissociation of the hybrid by treatment with imidazole citrate was the same as with dimeric phosphorylase *a*.

A comparison with *b* dimer indicates that the *b*-*a* hybrid is much less dependent on AMP for activity, since the hybrid was about 42% active without AMP (experiment 2). In both experiments 1 and 2, the maximum activity of the hybrid enzymes with AMP approached that of the nonphosphorylated or fully phosphorylated enzymes.

Phosphorylase *b* is competitively inhibited by glucose-6-P with respect to glucose-1-P. Inhibition is allosterically counteracted by AMP. Thus, with 100 mM glucose-1-P and 1 mM AMP, little or no inhibition of phosphorylase *b* occurs with glucose-6-P. Phosphorylase *a* is not at all inhibited by 5 mM glucose-6-P (21). The AMP-independent activity of matrix-bound phospho-dephosphohybrids, in contrast to phosphorylase *a* or *b* under these assay conditions, was inhibited 47% by 5 mM glucose-6-P. These properties distinguish the hybrid enzyme from phosphorylase *a* (1).

In order to find the direction of induction, experiment 3 was performed. Matrix-bound monomer *b*, which contained pyridoxal-P, was hybridized with inactive soluble subunits of pyridoxal-P ester phosphorylase *a*. The *a* subunit of pyridoxal-P ester phosphorylase induced activity in the matrix-bound *b* subunit, because only phosphorylase *b* has an absolute requirement for AMP for activity. The small AMP-independent activity of the hybrid enzyme could have been due to traces of unresolved holophosphorylase.

Hybrid phosphorylases containing active and inactive subunits

Phosphorylase derivatives containing stoichiometric amounts of pyridoxal-P ester or pyridoxal are inactive (18, 22). The directed induction of activity was therefore further studied with *a* monomers containing these inactive cofactor analogs. In experiments 1 and 2b of Table 3, the cofactors were covalently attached by reduction of the azomethine bond to the phosphorylase protein. This was necessary in order to prevent exchange of active and inactive cofactors (see ref. 18). A comparison of experiments 1, 2a, and 2b shows similar interactions for Sepharose-bound native and reduced phosphorylase *a* and their hybrid derivatives. Thus, the directed induction of activity by an intrinsically inactive subunit is not a peculiar property of reduced phosphorylase. Hybrids made from reduced and nonreduced subunits did not fully regain the activity of the original dimer (experiments 1 and 2b, Table 3), because reduced phosphorylase preparations are more readily denatured by ClHgBz than non-reduced phosphorylases. The important point of experiments 2a and 2b is that the interaction between matrix-bound *a* monomers with inactive soluble monomers elicited activity only in the potentially active subunit (see also: experiment 3, Table 2).

For determination of the specificity of subunit interactions in phosphorylase the experiments in Table 4 were performed.

† SDS can be removed nearly completely from Sepharose-bound *b* monomers by repeated washes with 3% serum albumin in 50 mM glycerol-P buffer (pH 7.0) followed by electrophoresis. However, the active dimer reconstituted by addition of soluble *b* subunits was still less stable than the native matrix-bound phosphorylase. It very slowly (1-2 hr) dissociated under assay conditions and lost activity.

Experiment 1 shows that induction of activity with an intrinsically inactive *a* subunit containing covalently bound pyridoxamine remained below the theoretically expected activity of 50% of the active sites. The scatter of the data was greater in these experiments than in the preceding experimental series suggesting that the pyridoxamine phosphorylase *a* hybrid was less stable than the corresponding hybrid formed with the subunit of pyridoxamine-P ester (experiment 2b, Table 3). Ionic strength, buffer ions, pH, and most importantly, temperature drastically affect subunit interactions in phosphorylase (6, 18, 23, 24, 29). These influences have not yet been studied with matrix-bound phosphorylase. Other experiments have shown that at temperatures above 20°, pyridoxal phosphorylase *b* has a more labile quaternary structure than holophosphorylase *b* or other analog reconstituted phosphorylases. The least stable structure was apophosphorylase *b* (18). This agrees with the results of hybridization experiments with apophosphorylase *b* monomers.

Experiment 2 in Table 4 indicates that no more than about 25% of the matrix-bound apomonomers had established contact with the subunit that contained pyridoxamine-P. This may be calculated from the difference between the activity of the holo-dimer *b* and that of the hybrid-dimer *b* after reconstitution with pyridoxal-P (0.60 against 0.14). If the latter activity of 0.14 is taken as baseline, one finds that the apophosphorylase *b* monomers in contact with the pyridoxamine-P monomers formed a hybrid enzyme with the expected activity, i.e., about 50% of the activity of the reconstituted *b* dimer (0.063 against 0.14). Experiments 3a and 3b show that induction of activity requires interaction between homologous *b* or *a* subunits, since other proteins known to form complexes with phosphorylase *b* and *a* were ineffective (25, 26, and unpublished data).

DISCUSSION

Chan has covalently attached rabbit skeletal muscle aldolase to activated Sepharose (15). The monomers covalently linked to the matrix showed about one-third the specific activity of the original Sepharose-bound tetramer. Graves *et al.* (24) reported that monomers formed after treatment of phosphorylase *b* reduced by NaBH₄ with 7% formamide likewise retained activity. Our evidence suggests that matrix-bound monomeric phosphorylase has little if any intrinsic activity, but activity appears upon noncovalent interaction with another subunit. Dimeric phosphorylase containing the active cofactor in one subunit and an inactive analog of pyridoxal-P (modified at the 5'-phosphate group or lacking the 5'-phosphate group) in the other subunit exhibits activity of only one subunit. Thus, phosphorylase has one active center per monomer, but the expression of activity of this single site requires interaction with another homologous subunit. An inactive subunit carrying a chemically modified cofactor is fully capable of eliciting activity in the potentially active subunit, but cannot itself become active. Thus, the intrinsically inactive subunit acts like a "regulatory" subunit.

We have chemically modified the cofactor that is essential for activity rather than the apoprotein. This is probably preferable, provided the inactive subunit that carries the analog is structurally complementary to the active holoenzyme. Structural complementarity was indicated by the extent of induction. The fact that phosphorylase subunits containing analogs of pyridoxal-P (which are themselves inactive) can induce activity argues for an additional role of the cofactor aside from that of a structural determinant. A

possible participation of one of the protonatable groups of pyridoxal-P (the 5'-phosphate group, $pK_2 = 6.2$) in the reaction catalyzed by glycogen phosphorylases has been discussed (18, 27).

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