Subunit Interactions and Their Relationship to the Allosteric Properties of Rabbit Skeletal Muscle Phosphorylase *b**

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ABSTRACT: Work was initiated to clarify the role of subunit interactions in the allosteric transitions of rabbit skeletal muscle phosphorylase *b*. When four SH groups per mole of phosphorylase dimer *b* are blocked by treating the enzyme with 5,5′-dithiobis(2-nitrobenzoic acid), activity is reduced by 50–60% and the characteristic sigmoidal activity response of the enzyme to the modifier 5′-adenosine monophosphate becomes hyperbolic. Release of the thionitrophenylate groups by dithiothreitol was complete in 3 min. The protein had rearranged itself in 30 min to a quarternary structure resembling that of the native enzyme. However full activity and allosteric kinetics were recovered only after hours. The fully reactivated enzyme was active to a considerable extent in the absence of 5′-adenosine monophosphate. The 5,5′-dithiobis(2-nitrobenzoic acid) derivatives of phosphorylase *b* could be separated by sucrose density gradient centrifugation into three molecular species. A monomeric species with two of its SH groups blocked per mole was completely devoid of activity. A dimeric species with up to three of its SH groups blocked per mole was fully active but had lost its allosteric activity response to 5′-adenosine monophosphate. A third species contained aggregated material with a molecular weight greater than dimer *b*. This material had two (or three) of its SH groups blocked (per mole of dimer *b*) but had a much lower specific activity than the dimer *b* fraction. It was concluded that there exist two (and possibly three) contact regions in the monomeric subunit. One seems responsible for the allosteric properties of phosphorylase *b*. This contact region is weakened in the dimer *b* by reaction of only one SH group per each monomer. The forces which are responsible for the contact between monomers in the dimeric structure are stronger. One additional group must be blocked per monomer *b* in order that dissociation of dimer *b* into two monomers can occur. It is proposed, in accordance with Chignell *et al.* (Chignell, D. A., Gratzer, W. B., Valentine, R. C. (1968), *Biochemistry 7, 1082*), that only when contact between these regions in the protein is weakened (for example, by reaction of SH groups with 5,5′-dithiobis(2-nitrobenzoic acid), irregular association may occur yielding aggregates of higher order which have a structure different from that of native tetramer *b*. These aggregates are largely inactive. After removal of the prosthetic group, pyridoxal 5-phosphate, from phosphorylase *b* a protein is formed which resembles the molecular species that are formed by treatment of phosphorylase *b* with 5,5′-dithiobis(2-nitrobenzoic acid). Apophosphorylase *b* and the holoenzyme bind 5′-adenosine monophosphate about equally well. However, heterotropic cooperativity between the binding sites for 5′-adenosine monophosphate and glucose 1-phosphate is abolished and homotropic cooperativity between the binding sites for 5′-adenosine monophosphate is greatly weakened. Binding of pyridoxal and of 5′-deoxypyridoxal to the apoprotein restores a quarternary structure quite similar to that of the holoenzyme and reestablishes homo- and heterotropic cooperativity to an extent qualitatively similar to that found with the native holoenzyme. Although the 5′-deoxypyridoxal and the pyridoxal phosphorylase *b* proteins meet the structural requirements for the expression of allosteric properties, the only active protein is that containing pyridoxal 5-phosphate. Therefore a different structure of the prosthetic group is required to establish subunit interactions and to make the protein function as a catalyst. A possible role of the 5-phosphate group of pyridoxal 5-phosphate in catalysis by glycogen phosphorylase is therefore suggested. Results of a correlational study of the effect of temperature on quarternary structure and binding of 5′-adenosine monophosphate to apophosphorylase *b*, the holoenzyme, and the 5′-deoxypyridoxal derivative of phosphorylase *b* also point to similarities between phosphorylase *b* and its 5′-deoxypyridoxal analog. Apophosphorylase *b* differs also with respect to these parameters from phosphorylase *b* and the 5′-deoxypyridoxal analog. Exposure to cold is another means by which the subunits in the phosphorylase *b* proteins can be induced to associate.

Work on a model for the allosteric transitions of skeletal muscle phosphorylase *b* directed our attention to the role of subunit interactions and their relationship to the allosteric properties of rabbit muscle phosphorylase *b*. (*cf.* L. L. Kastenschmidt, J. Kastenschmidt, and E. Helmreich, unpublished

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data; see also Helmreich, 1967; Kastenschmidt *et al.*, 1967).

Transitions of protomers (T) to protomers (R) in an oligomeric protein[1] need not lead *per se* to changes in quarternary structure. For example, yeast glyceraldehyde 3-phosphate dehydrogenase is likewise a tetramer (Harris and Perham, 1965). The allosteric T $\rightleftharpoons$ R transitions of this enzyme are fully concerted as shown in the elegant kinetic study of Kirschner *et al.* (1966), but in the course of these transitions there are no detectable changes in the quarternary structure of the holo- or apoenzyme. This is in contrast to phosphorylase *b*. Dimers *b* can be induced by the allosteric modifier 5′-AMP[2] under certain conditions to associate to tetramers (Kent *et al.*, 1958). The reverse also occurs. The slow dissociation of tetramers of phosphorylase *a* (and phosphorylase *b;* B. E. Metzger, unpublished results) by glycogen may serve as an example (Wang *et al.*, 1965; Metzger *et al.*, 1967, 1968).

Furthermore it is known that muscle phosphorylases *a* and *b* can, under certain conditions (*i.e.*, upon removal of pyridoxal-5-P or upon blocking of SH groups), dissociate into monomeric subunits (Illingworth *et al.*, 1958; Madsen and Cori, 1956). These earlier observations seemed to offer attractive possibilities for an experimental approach to the study of subunit interactions with this enzyme. More specifically, an attempt was made to block the transition from one conformational state to another by uncoupling the interacting subunits. Two conditions were chosen. The first approach involved the use of thiol-reactive agents. In this study DTNB (Ellman, 1959) was used which forms mixed disulfides by means of disulfide interchange with certain SH groups of native phosphorylase *b* (Damjanovich and Kleppe, 1966; Buc and Buc, 1968). Secondly, the allosteric behavior and the quarternary structure of apophosphorylase *b* was compared by means of binding studies and ultracentrifugal analyses with the corresponding parameters for active phosphorylase *b* and for the phosphorylase *b* protein after it was recombined with the pyridoxal 5-phosphate analogs, pyridoxal and 5′- deoxypyridoxal. The results suggest the existence of several contact surfaces on the monomers which are involved in the formation of dimers, tetramers, and aggregates of higher order. The results bear also on the enigmatic role of pyridoxal 5-phosphate in phosphorylase. After this work was completed a paper by Chignell *et al.* (1968) appeared which is pertinent to the problem under discussion.

Methods

Phosphorylase b was prepared from frozen rabbit muscle using the procedure of Fischer and Krebs (1958). The enzyme was recrystallized at least three times prior to use. In the ultracentrifuge phosphorylase *b* (4.5 mg/ml) gave a single homogeneous peak at 20° with a s_{20} value (uncorrected) of 8.3 S (*cf.* Brown and Cori, 1961). Crystal suspensions were stored at 2–4° under toluene vapor. If necessary the enzyme was freed from 5′-AMP. For this purpose, phosphorylase *b* (40–70 mg/ml) was passed over a Sephadex G-25 column (0.9 × 27 cm). The column was preequilibrated with 50 mM sodium glycerol-P–2 mM EDTA–1 mM 2-mercaptoethanol buffer (pH 6.8). Following Sephadex treatment of phosphorylase *b*, the absorbancy ratios $A_{260\,m\mu}:A_{280\,m\mu}$ of the enzyme ranged from 0.54 to 0.53. Protein concentrations were determined by absorbance measurements at 280 mμ using an absorbancy index ($1\%\times cm^{-1}$) of 13.2. This value was derived from the following data. Phosphorylase *b*, several-times recrystallized and free of 5′-AMP ($A_{260\,m\mu}:A_{280\,m\mu}$ 0.54), was exhaustively dialyzed against 50 mM phosphate–1 mM 2-mercaptoethanol buffer (pH 6.8). The nitrogen content of several aliquots of this preparation was determined by a micro-Kjeldahl procedure (Kabat and Mayer, 1961). It was found that an enzyme solution with an absorbancy at 280 mμ of $1.250\times cm^{-1}$ contained 0.16 mg of nitrogen/ml. From these data and the data of Velick and Wicks (1951) for the amino acid composition of rabbit skeletal muscle phosphorylase *b*, one obtains an absorbancy index ($1\%\times cm^{-1}$) of 12.9. If one uses the newer data of Appleman *et al.* (1963) for the amino acid composition of rabbit muscle phosphorylase *b*, one obtains a value of 13.2. These values agree with those reported recently for rabbit muscle phosphorylase *b* by Buc and Buc (1968) (*A* 13.5) and with the absorbancy index (*A* 12.8) recently determined with purified frog skeletal muscle phosphorylase *b* (Metzger *et al.*, 1968). The newer values are higher than the absorbancy indices previously used in this and other laboratories[3] (*A* 11.7, Velick and Wicks, 1951; *A* 11.9, Appleman *et al.*, 1963).

In some instances phosphorylase *b* concentrations were determined with the method of Lowry *et al.* (1951) using phosphorylase *b* as reference standard.

[1] The nomenclature introduced by Monod *et al.* (1965) is used. For further information this and other papers by Monod and colleagues may be consulted. Relevant references may be found in the paper by Helmreich *et al.* (1967).

[2] Abbreviations used that are not listed in *Biochemistry 5*, 1446 (1966), are: pyridoxal-5-P, glucose-1-P, glucose-6-P, glycerol-P, etc., are pyridoxal 5-phosphate, α-D-glucose 1-phosphate, glucose 6-phosphate, DL-β-glycerol phosphate, etc.; 5,5′-dithiobis(2-nitrobenzoic acid) is DTNB (Ellman's reagent); 2,3-dihydroxy-1,4-dithiolbutane is dithiothreitol (Cleland, 1964); *p*-hydroxymercuribenzoate is PMB.

[3] In the meantime a considerable effort was made to verify the new values for the absorbancy indices by measurements based on dry weights. Phosphorylase *b* was first passed through a charcoal column and subsequently through a Sephadex G-25 column equilibrated with 5 mM phosphate buffer (pH 6.8). The absorbancy of the protein solutions (2–4 mg/ml) at 280 mμ was either determined directly or after dilution in 2- or 10-mm path cells. The protein solutions were brought to dryness in a freeze dryer at 42–44° *in vacuo*. Usually after 48 hr constant dry weights were obtained. An extensive series of measurements gave a linear relationship of optical density to protein concentrations and yielded values for *A* ($1\%\times cm^{-1}$) in the limits from 12.1 to 12.5. Similar observations were made independently in Dr. D. J. Graves' laboratory (personal communication by Dr. Wang). The discrepancy between the values for *A* based on the amino acid compositions and based on dry weights remains to be explained.

TABLE I: The Role of SH Groups in the Maintenance of Quarternary Structure and Activity of Phosphorylase *b*.[a]

Enzyme Fractions	Moles of SH Groups Blocked/Mole of Monomer[b]	Activity (μmoles mg^{-1} min^{-1})		
		Before Reduction	After Reduction	
			+Pyridoxal-5-P	−Pyridoxal-5-P
Monomer	2.2–2.8	0	32	32
Dimer	1.1–1.6	34	38	38
Aggregates	1.0–1.8	1.4–6.3	37	37

[a] The enzyme fractions assayed were obtained by sucrose density gradient centrifugation of phosphorylase *b* treated with DTNB as described in Methods. The preparation contained an average of four nitrophenylate groups in mixed disulfide linkages. Results of three separate experiments of the type shown in Figure 2 are given. The range for the number of mixed disulfides formed is indicated. The range of specific activities is also shown for the aggregates, since their activity varied from preparation to preparation. Phosphorylase *b* activity was measured before and after reduction of the mixed disulfides with dithiothreitol. Reduction of the DTNB derivatives of phosphorylase *b* was carried out by incubating the enzyme with 1 mM dithiothreitol at 23° in 50 mM sodium glycerol-P buffer (pH 6.8) for 6 hr. Following incubation for this period of time the maximal extent of activity was usually recovered (see Table II). When pyridoxal 5-phosphate was added during reduction its concentration was 1×10^{-5} M which is about 50–100 times the molar concentrations of the enzyme fractions used. Activities were measured under standard assay conditions at 30° (see Methods). [b] A molecular weight of 90,000 was used for calculations.

Apophosphorylase b. Phosphorylase *b*, about 4 mg/ml, free of 5′-AMP was dissolved in equimolar (50 mM) 2-mercaptoethanol–sodium glycerol-P buffer (pH 7.0). Pyridoxal 5-phosphate was removed from the protein as described by Shaltiel *et al.* (1966). The apophosphorylase *b* was precipitated with $(NH_4)_2SO_4$. The precipitate was dissolved in 50 mM sodium glycerol-P–50 mM 2-mercaptoethanol buffer (pH 7.0). $(NH_4)_2SO_4$ was removed by passing the protein over a Sephadex G-25 column which was equilibrated with the buffer used for binding studies (see below).

Reconstitution Experiments. Reconstitution of the apophosphorylase *b* protein was carried out at 37° either with the natural prosthetic group, pyridoxal 5-phosphate, or with the vitamin B_6 analogs, pyridoxal and 5′-deoxypyridoxal, respectively. The experimental conditions were those described by Hedrick *et al.* (1966). The apoprotein preparations and the reconstituted phosphorylase *b* preparations were analyzed for pyridoxal 5-phosphate, pyridoxal, and 5′-deoxypyridoxal, respectively. These analyses involved precipitation and release of the proteins from pyridoxal 5-phosphate, or from the analogs by 0.1 N perchloric acid as described by Baranowski *et al.* (1957). Pyridoxal 5-phosphate, 5-deoxypyridoxal, and pyridoxal were determined spectrophotometrically at 295 mμ using absorbancy indices of 6250 (*cf.* Shaltiel *et al.*, 1966), 6500 (*cf.* Heyl *et al.*, 1953), and 8600 l. mole^{-1} (Peterson and Sober, 1954), respectively. The recovery of all three pyridoxal compounds ranged from 2.2 to 1.8 moles per 185,000 daltons of phosphorylase *b*.

Reduction of Phosphorylase b with $NaBH_4$. The procedure of Graves *et al.* (1965) was used. After reduction was completed, the enzyme was precipitated by the addition of $(NH_4)_2SO_4$. $(NH_4)_2SO_4$ was removed by passing the enzyme dissolved in buffer over Sephadex G-25. The reduced enzyme was crystallized by the addition of 10 mM magnesium acetate and 1 mM 5′-AMP. The efficacy of the procedure was checked by determining how much of the pyridoxal 5-phosphate could be released after denaturation of the enzyme with perchloric acid. Only about 0.1% of the total pyridoxal 5-phosphate content of the reduced enzyme was lost. The reduced enzyme retained about 70% of the activity of unreduced phosphorylase *b* measured under standard assay conditions.

Treatment of Phosphorylase b with 5,5′-Dithiobis(2-nitrobenzoic Acid). Mixed disulfides were formed by disulfide interchange between the SH groups of phosphorylase *b* and DTNB (*cf.* Ellman, 1959). For this purpose, usually about 10 mg of phosphorylase *b* free of 5′-AMP was dissolved in 0.9 ml of 50 mM sodium glycerol-P–2 mM EDTA buffer (pH 6.8) and 0.1 ml of a 10 mM solution of DTNB in the same buffer was added. The molar concentrations of enzyme and DTNB in the reaction mixture were 5.4×10^{-5} and 1×10^{-3} M, respectively, resulting in a 18.5-fold molar excess of DTNB. Molar concentrations of phosphorylase *b* were calculated in these and in all other experiments on the basis of a molecular weight of 185,000 daltons (*cf.* Seery *et al.*, 1967; Buc and Buc, 1968; DeVincenzi and Hedrick, 1967; Metzger *et al.*, 1968). The reaction mixture was incubated usually for about 7 hr at room temperature (23°). The molar concentration of the DTNB-reactive SH groups was determined spectrophotometrically by following the change in absorbancy at 412 mμ due to the formation of the thiophenylate anion. An extinction coefficient (l. mole^{-1} cm^{-1}) of 13,600 at pH 6.8 was used (Ellman, 1959). A blank for the unreacted DTNB was deducted. Following reaction

of phosphorylase *b* with DTNB, the reaction mixture was passed over Sephadex G-25 equilibrated with 50 mM sodium glycerol-P–2 mM EDTA buffer (pH 6.8). Fractions usually containing about 9 mg of protein/1.5 ml were recovered from the column. The DTNB-treated enzyme was used for kinetic and ultracentrifugal studies. For other experiments the DTNB-derived enzyme was further fractionated by sucrose density gradient centrifugation (Martin and Ames, 1961).

Sucrose solutions (30 and 10%, w/v) were prepared with 50 mM sodium glycerol-P–2 mM EDTA buffer (pH 6.8). Aliquots (2.5 ml) of these solutions were layered into 0.5 × 2 in. cellulose nitrate tubes with a device for making linear gradients (*cf.* Martin and Ames, 1961). Samples (1.4 mg in 0.2 ml) of a phosphorylase *b* preparation treated with DTNB as described above and a sample of the reference enzyme (about 0.1 mg/0.01 ml) were mixed and layered on top of the sucrose gradient. Crystalline rabbit muscle aldolase was used as a reference enzyme. Its molecular weight was taken as 150,000 daltons (Penhoet *et al.*, 1967). Centrifugation was carried out for 17 hr in a Spinco Model L preparative ultracentrifuge at 18–20°. A swinging-bucket rotor, SW-39, and a rotor speed of 37,000 rpm were used. After completion of separation, the tubes were punctured with a 27-gauge needle to which was attached a 7-in. piece of fine polyethylene tubing. A total of 37 fractions each containing 0.14 ml or 10 drops of the solution was collected. As judged by their sedimentation behavior with reference to that of aldolase, tubes 6–10 contained a heavy fraction, tubes 14–18 phosphorylase *b* dimer, and tubes 20–25 phosphorylase *b* monomer. The three fractions were each pooled and the number of the SH groups which had reacted with DTNB was determined in each of the three fractions. To aliquots (0.49 ml) obtained from each fraction, 0.01 ml of a 50 mM dithiothreitol solution was added. The addition of dithiothreitol (Cleland, 1964) causes the reduction of the mixed disulfide and results in the release of the blocking thionitrophenylate group. Release was followed by measuring the increase in absorbancy at 412 mμ. Enzyme activity was determined before and after incubation at 23° with dithiothreitol for the times specified in the legends to Table I and II and Figure 5. Aliquots of each fraction (or from each tube) were diluted with 50 mM sodium glycerol-P–2 mM EDTA buffer (pH 6.8) in order to make the enzyme concentrations about equal in each sample and suitable for enzymatic assays. The final volume was 1 ml. Aliquots (0.05 ml) were withdrawn for phosphorylase *b* or aldolase activity assays. Protein concentrations were determined prior to the addition of dithiothreitol.

Enzyme Activity Measurements. Activity assays of phosphorylase *b* were carried out in the direction of glycogen synthesis. For routine assay, phosphorylase *b* (20–40 μg/ml) was incubated at 30° with 1.5 mM 5′-AMP, 0.5% glycogen (which corresponds to 2.5 × 10^{-3} M glucosyl groups at the nonreducing end of the chains), 37.5 mM glucose-1-P, 25 mM sodium glycerol-P, 1 mM EDTA, and 0.5 mM mercaptoethanol buffer (pH 6.8). P_i released in the reaction was measured by the method of Fiske and Subbarow (1925). First-order velocity constants were determined as described by Cori *et al.* (1943). Activity of phosphorylase *b* was expressed in micromoles of product formed per minute per milligram of enzyme. The specific activity of the native phosphorylase *b* preparations determined under these standard assay conditions ranged from 50 to 60 μmoles of P_i formed per min per mg of enzyme. The reaction mixture for kinetic measurements is described in the legend to Figure 5. The ionic strength of the solutions was $\Gamma/2 = 0.13$. When phosphorolysis of glycogen was measured, a coupled enzyme assay with phosphoglucomutase and glucose 6-phosphate dehydrogenase was used (*cf.* Helmreich and Cori, 1964a). For measurements of fructose 1,6-diphosphate aldolase activity, the modification by Bruns (1954) of the method of Sibley and Lehninger (1949) was used.

TABLE II: Time Course of Recovery of Activity of DTNB-Treated Phosphorylase *b* Following Reduction.[a]

Incubn with Dithiothreitol (min)	Act. (μmoles mg^{-1} min^{-1})	
	Phosphorylase *b* (unfractionated)	Monomer *b*
0	18	0
1	17	3.9
3	19	
4		2.6
10	21.0	10.4
20	24.5	
60	33.5	
120	39.0	
240	42.0	
300		42.0

[a] A phosphorylase *b* preparation (sp act. 63 μmoles/mg per min) was treated with DTNB as described in Methods. It contained four nitrophenylate groups per mole of dimer *b*. An aliquot of the enzyme derivative was tested for activity under routine assay conditions at 30° following incubation with 1 mM dithiothreitol at 23° for the times indicated. Another part of the enzyme preparation was fractionated by sucrose gradient centrifugation (see Figure 2) into the monomer fraction. This fraction was likewise assayed following incubation with 1 mM dithiothreitol (see also legend to Table I).

Binding of 5′-AMP. The binding of 5′-AMP to muscle phosphorylase *b* was measured by means of the gel filtration method of Hummel and Dreyer (1962) as modified by Fairclough and Fruton (1966).

The design of the Sephadex G-25 columns and the general procedure used for binding studies were similar to those described in detail in the paper of Fairclough and Fruton (1966). Four columns of the dimensions 0.5 × 115 cm were filled with Sephadex G-25 and placed within a constant-temperature jacket. Water was circulated through the jacket at the desired tem-

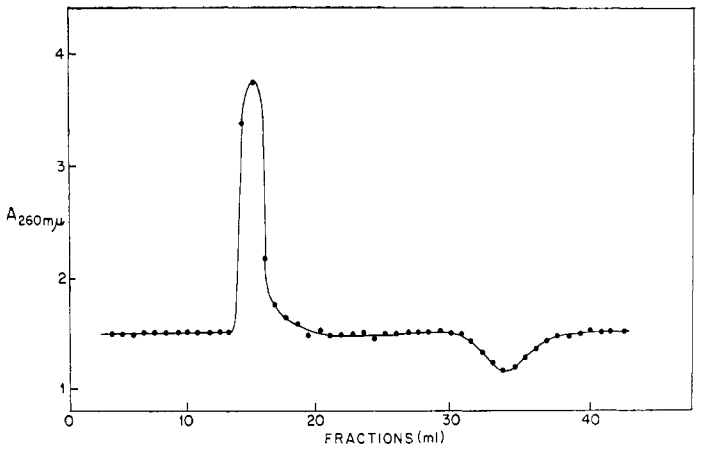

FIGURE 1: Elution diagram of phosphorylase *b* and 5′-AMP from a Sephadex G-25 column (see Methods). The concentrations of phosphorylase *b* and 5′-AMP are 1.3×10^{-4} and 1×10^{-4} M, respectively. The temperature was 23°.

TABLE III: Binding of 5′-AMP to Apophosphorylase *b* and to $NaBH_4$-Reduced Phosphorylase *b*.[a]

Additions (mM)	Holophosphorylase *b*, $\Gamma/2 = 0.13$, K_{dissn} (M × 10^4)	Apophosphorylase *b*		$NaBH_4$-Reduced Phosphorylase *b*, $\Gamma/2 = 0.13$ K_{dissn} (M × 10^4)
		$\Gamma/2 = 0.13$, K_{dissn} (M × 10^4)	$\Gamma/2 = 0.47$, K_{dissn} (M × 10^4)	
None	3.7 ± 0.23	5.3 ± 1.2	10.0	3.5 ± 1.0
Glucose-1-P (50)	0.73 ± 0.22	5.7 ± 1.7		1.3 ± 0.2
Glucose-1-P (200)			9.6	

[a] The experiments were carried out at 23° in 10 mM sodium glycerol-P, 2 mM EDTA, and 1 mM 2-mercaptoethanol buffer (pH 6.8). The ionic strength was brought to $\Gamma/2 = 0.13$ or to $\Gamma/2 = 0.47$ by addition of KCl. With the exception of the experiments at $\Gamma/2 = 0.47$, the average of three to five experiments with the standard error of the mean is shown. The concentration of 5′-AMP was 0.1 mM. The concentrations of holo-, apo-, and $NaBH_4$-reduced phosphorylase *b* were 18, 14.3, and 12.0 mg/ml, respectively.

perature by means of a constant-temperature bath (Forma-Temp).

Phosphorylase *b* (6–25 mg) was usually dissolved in 1 ml of 50 mM sodium glycerol-P–2 mM EDTA–1 mM 2-mercaptoethanol buffer (pH 6.8) containing the same concentration of 5′-AMP as the equilibration buffer. As stated in the legends to Tables III–V, in addition to 5′-AMP, glucose-1-P, glucose-6-P, and KCl were added in certain experiments. The columns were equilibrated and eluted with the same sodium glycerol-P buffer (including the same concentrations of 5′-AMP and of other ligands). The eluate from the columns was collected in serial fractions of about 1 ml each. The flow rate over the column varied with temperature. It ranged from 12 ml/hr at 4° to 35 ml/hr at 34°. The 5′-AMP concentration in the eluate was determined by absorbancy measurements at 260 mμ using cells with 2- or 10-mm light paths. A molar extinction coefficient for 5′-AMP of 15.4×10^3 cm^{-1} was used. The absorbancy was read against the buffer solution in a reference cuvet. With the type of columns used and the amount of protein applied, the peak and the trough in the elution diagram were well separated from each other. The protein appeared after 11 ml of the eluate was collected and the trough appeared between 30 and 38 ml of the eluate. A typical elution diagram is shown in Figure 1. The ionic strength of the solution was $\Gamma/2 = >0.13$, and the protein concentration did not exceed 25 mg/ml. An influence of the Donnan equilibrium was therefore neglected. Control experiments were carried out with 2′,3′-AMP because this nucleotide does not bind to phosphorylase *b* (Brown and Cori, 1961).

Calculations. The amount of 5′-AMP was determined in each experiment from the area above the trough. The absorbancy of the eluted fluid just preceding and immediately following the trough was a measure of the base-line absorbancy from which the concentration of free 5′-AMP could be calculated. From the differences in the absorbancies between the base line and the trough, one calculates the amount of bound 5′-AMP. An equation given by Fairclough and Fruton (1966) was used.

$$\mu\text{moles of bound 5′-AMP} = \frac{(A_{basal} - A(\text{fraction in trough})) \times \text{ml of (fraction in trough)}}{A(\text{M} \times 10^{-3})}$$

Knowing the concentration of free and bound 5′-AMP,

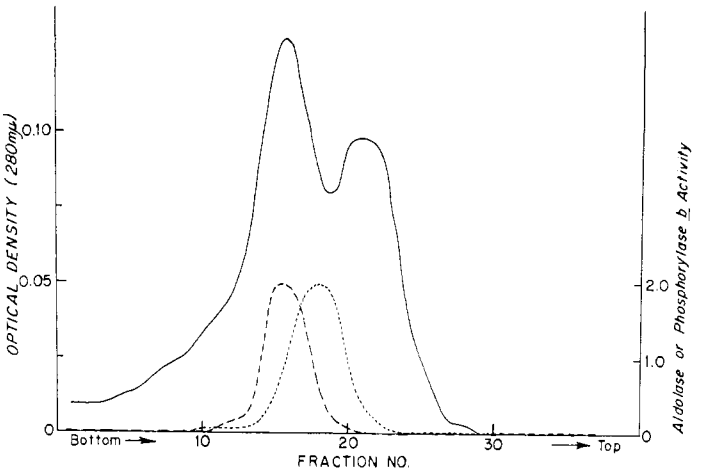

FIGURE 2: Fractionation of DTNB-treated phosphorylase *b* by sucrose density gradient centrifugation. A phosphorylase *b* preparation containing four nitrophenylate groups in mixed disulfide linkages was used. For experimental details, see Methods. On the right-side ordinate, aldolase or phosphorylase *b* activity is registered. Aldolase activity is expressed in terms of μmoles of fructose 1,6-diphosphate cleaved in each fraction × min^{-1}. Phosphorylase *b* activity is expressed in terms of μmoles of P_i formed in each fraction x min^{-1}. Solid lines represent absorbancy measurements at 280 mμ. Broken lines represent activity measurements: (·····) aldolase, (– – – – – –) phosphorylase *b*.

the data were then plotted according to the equation $r/C_{AMP} = K_{assn}(n - r)$, proposed by Scatchard (1947) and Klotz (1953), where r represents the average number of 5′-AMP molecules bound to each molecule of phosphorylase *b*, n is the apparent number of available binding sites on each molecule of enzyme, and C_{AMP} is the concentration of free AMP.

Plots of r/C_{AMP} *vs.* r are shown in Figure 6, which will be described later on. The intercept on the abscissa is equal to K_{assn} ($K_{assn} = 1/K_{dissn}$). Binding plots for 5′-AMP and phosphorylase *b* and its derivatives are curvilinear. In this case the value for K_{dissn} can be estimated by drawing a tangent to the linear part of the slope but an additional method of calculation may also be used. The curves are first converted into linear slopes by plotting the data in the form of log $r/(n - r)$ *vs.* log C_{AMP}. The lines were drawn by the method of least squares. The concentration required for half-maximal saturation may then be estimated by extrapolation to the abscissa (log C_{AMP}) from the intercept of the lines with the zero axis (on the log $r/(n - r)$ ordinate). The slope also defines the interaction coefficient of Brown and Hill (1922). Both methods were used.

Binding of Glycogen. The binding of glycogen to apophosphorylase *b* was studied using the ultracentrifugal separation technique of Madsen and Cori (1958). The same sample of phytoglycogen which was used by these authors was also used in the experiments reported in this paper.

Ultracentrifugal Analysis. Sedimentation velocity experiments were carried out in the Spinco Model E analytical ultracentrifuge using a single-sector cell with a 0.15-cm light path. The rotor speed was 59,780 rpm. Pictures were taken 4–40 min after the rotor had reached full speed. Temperature was maintained within ±0.3° of the temperature indicated. The schlieren diagrams were evaluated by calculating boundary movements from direct measurements with a Nikon Model 6C microcomparator. The percentage of the components with different sedimentation coefficients in associating-dissociating enzyme solutions was estimated from projections of the enlarged sedimentation patterns on paper. The area under the sedimentation peaks was determined with a planimeter (Keuffel and Esser Co.).

Materials. Frozen rabbit muscle was the source of phosphorylase *b*. The meat was obtained from Pel-Freeze Biologicals, Rogers, Ark. Rabbit liver glycogen was obtained from Mann Research Laboratories, Inc. It was freed from nucleotides as described by Helmreich *et al.* (1967). 5′-AMP was purified as described by Helmreich *et al.* (1967). DTNB was recrystallized from acetic acid prior to use. Glucose 6-phosphate dehydrogenase, phosphoglucomutase, and crystalline rabbit muscle fructose 1,6-diphosphate aldolase were products of Boehringer & Sons. The enzymes were dialyzed prior to use as described by Helmreich and Cori (1964a). Pyridoxal was obtained from the Sigma Chemical Co. Samples of 5′-deoxypyridoxal were generous gifts from Dr. Esmond S. Snell and from Dr. Walter B. Gall (Merck Sharp and Dohme Research Laboratories). Imidazole was purified as described previously (*cf.* Helmreich *et al.*, 1967).

Sephadex G-25 (coarse grade) was a product of Pharmacia and was obtained through Sigma Chemical Co. It was washed ten times with 0.1 M NaCl by resuspending and decanting. The columns were packed with the washed, decanted Sephadex gel by gravity to an equal height of about 110 cm. All other reagents were of the highest commercially available purity. Double-distilled water was used throughout.

Results

Quarternary Structure and Allosteric Properties of DTNB-Treated Phosphorylase b. It is known through the work of Damjanovich and Kleppe (1966, 1967) that there are two classes of SH groups in phosphorylase *b* which differ with respect to rates of reaction with DTNB. Two (to three) SH groups per dimer *b* react rapidly with DTNB causing little or no loss of activity. The remaining SH groups react much slower with DTNB and a decrease in activity occurs. We could now show that the reaction of four SH groups of the dimer *b* with DTNB markedly changes the quarternary structure and the allosteric properties of phosphorylase *b*. Changes in the quarternary structure of phosphorylase

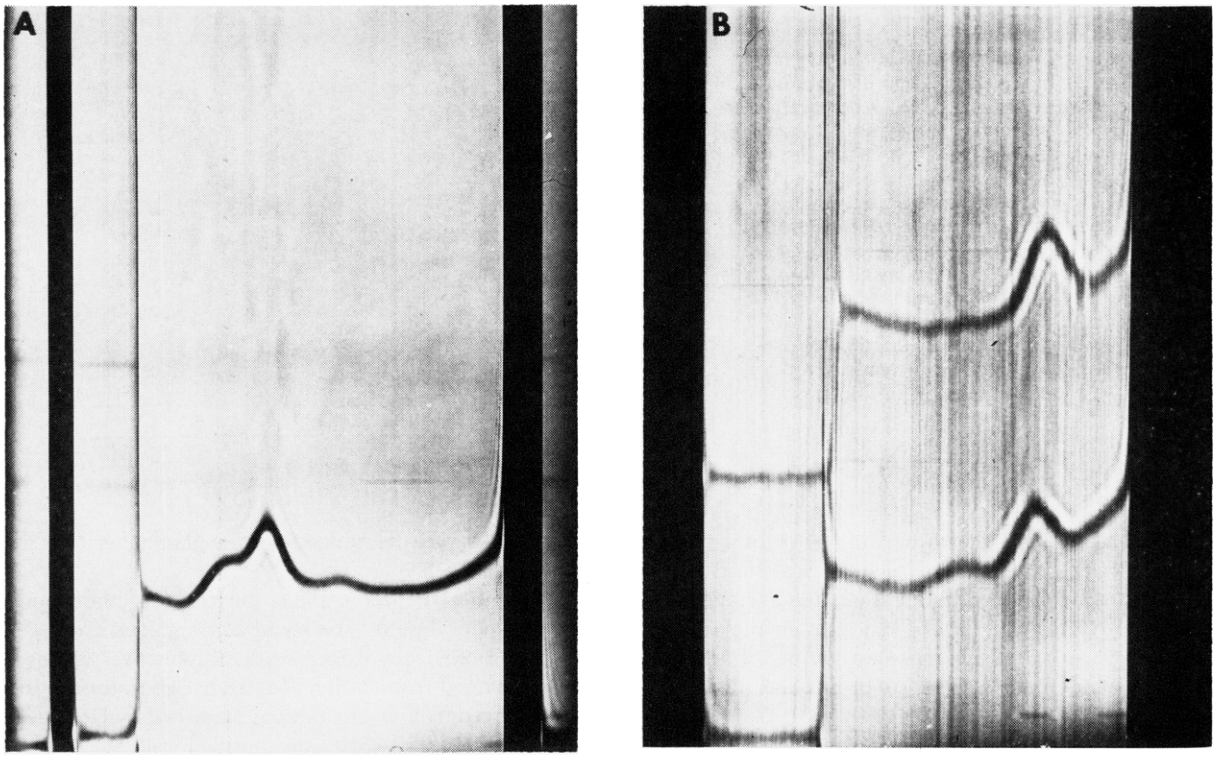

FIGURE 3: Sedimentation velocity measurements of DTNB-treated phosphorylase *b*. A phosphorylase *b* preparation containing four nitrophenylate groups in mixed disulfide linkages was used. In panel A, the enzyme (6.7 mg/ml) in 50 mM sodium glycerol phosphate–2 mM EDTA buffer (pH 6.8) was centrifuged at 23°. The picture shown was taken 30 min after the rotor had attained maximum speed. In panel B are shown experiments with another DTNB derivative of phosphorylase *b*. The same buffer system was used; however, to the preparation shown on top 1 mM dithiothreitol was added at the start of centrifugation. The concentration of protein was 7.26 mg/ml, the temperature was 23°. The pictures shown were taken 31 min after the rotor had reached maximum speed. Sedimentation in A and B was from left to right.

b upon treatment with DTNB were also reported recently by Buc and Buc (1968).

Phosphorylase *b* dimer containing on the average four mixed disulfides per mole can be fractionated by sucrose density gradient centrifugation into monomers, dimers, and polydisperse aggregates of higher molecular weight (molecular weight > dimer *b*) (Figure 2, Table I).

Monomeric Subunits. The data in Table I show that the monomeric species when compared with the other molecular species contains on the average one additional mixed disulfide per mole. There are a total of two (or three) SH groups on monomer *b* which have reacted with DTNB but only one (to about one and one-half) in the fractions containing dimer *b* or aggregated materials. When these two (to three) SH groups in the monomer *b* are linked to the nitrophenylate groups, an inactive monomer is obtained. Whether the loss of activity is a consequence of the dissociation of active dimer *b* to monomers which are *per se* inactive or if it is caused by blocking of SH groups which are essential for activity cannot be decided on the basis of these experiments. Other evidence which bears on that point will be discussed later. The results so far suggest only that there are one (possibly two) critical SH groups per monomer *b* which are involved directly or indirectly in the contact between monomers in dimer *b*. Reaction with DTNB weakens this interaction and consequently the dimer *b* dissociates into two monomers.

We assume that interaction between these contact sites is considerably stronger than the interactions that are responsible for the allosteric behavior of dimer b. In the following paragraph we present additional evidence to support this assumption. The latter, weaker interactions are probably also responsible for the association of dimer *b* to tetramer *b* (*cf.* L. L. Kastenschmidt, J. Kastenschmidt, E. Helmreich, unpublished data). The greater number of blocked SH groups in the monomer *b* following reaction with DTNB might be indicative of stronger interactions which involve additional SH groups in surface contact. However, aside from these interactions there must still exist a third contact region between monomers in phosphorylase *b*. Normally, we would not expect phosphorylase *b* with its SH groups intact to associate to its tetrameric form under the conditions of these experiments (*i.e.*, at an enzyme concentration of about 1.4 mg/ml in the absence of 5′-AMP and at 23°). We have carried out a control experiment using 1.84 mg of phosphorylase *b* and the above conditions. The native phosphorylase *b* gave only a single peak which corresponded to dimer *b* on sucrose density gradient centrifugation. However, after DTNB treatment a fraction of aggregated enzyme with a molecular weight greater than that of dimer *b* was found in the bottom fractions of the sucrose gradient (see Figure 2). This fraction had little activity and contained only about one mixed disulfide per monomer

(see Table I). The results obtained with native phosphorylase *b* and the low activity and unusual tendency of self-association of the DTNB-treated enzyme makes it very unlikely that this fraction of aggregated molecules represents tetramer *b*. The conclusion then is that there exists an additional surface contact between monomers and that interaction between these sites results in the association to molecular aggregates other than the normal tetrameric *b* structures. Contact between this third type of sites apparently is possible only after interactions between monomers are weakened, for example, by blocking of SH groups. Chignell *et al.* (1968) have reached a similar conclusion on the basis of their evidence.

A sedimentation velocity picture of phosphorylase *b* containing four blocked SH groups is shown in Figure 3A. It is obvious that the enzyme protein is a heterogeneous mixture of monomers ($s_{20,w}$ = 5.3 S), dimers ($s_{20,w}$ = 7.6 S), and aggregates ($s_{20,w}$ = >13.5 S). S values for the aggregated material are larger than that of tetramer *b* ($s_{20,w}$ = 13.0 S; Kent *et al.*, 1958).

The coupling reaction of the SH groups of phosphorylase *b* with DTNB was carried out with a fairly small molar excess of the reagent (see Methods). Under these conditions, the time of reaction is slow enough (measured in hours) so that one can follow the reaction conveniently by observing the change in absorbancy at 412 mμ and can stop the reaction when four SH groups per mole of the original dimer *b* have reacted with DTNB. The time course of reactivation of phosphorylase *b* following the release of the blocking group by dithiothreitol is shown in Table II. A few data on the reactivation of monomer *b* are also shown. From Figure 4 it can be seen that the thionitrophenylate group is released by reduction of the mixed disulfide with a half-time of less than 1 min. The complete recovery of activity is, however, a slow process (see Table II). Some active enzyme is re-formed soon after release of the blocking group, but complete return of activity takes several hours. For example, only after 4–6-hr incubation with dithiothreitol had the specific activity risen to about 38–42 μmoles of product formed/mg of enzyme per min (see Tables I and II). This is quite comparable with, although somewhat lower, than the usually observed specific activity of native phosphorylase *b* preparations (see Methods). The specific activity did not increase much when the enzyme was exposed to dithiothreitol for up to 24 hr.[4] The addition of pyridoxal 5-phosphate neither affected the extent nor the rate of reactivation of DTNB-treated phosphorylase *b* by dithiothreitol (Table I).

The re-formation of the original dimeric structure of phosphorylase *b* following reduction by dithiothreitol was followed in the analytical ultracentrifuge (Figure 3B). Phosphorylase *b* with four mixed disulfides per mole of dimer *b* was reduced with dithiothreitol as described in the legends to Tables I and II. The enzyme was centrifuged at 23° and the first pictures in which

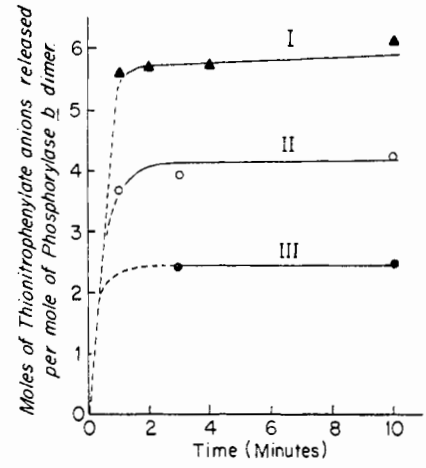

FIGURE 4: Time course of release of thionitrophenylate anions. Phosphorylase *b* was treated first with DTNB as described in Methods. Unfractionated phosphorylase *b* (II) (–○–○–○–) or phosphorylase *b* fractionated by sucrose density gradient centrifugation (see Figure 2 and Methods) into monomers (I) (–▲–▲–) and aggregates (III) (–●–●–●–) were then exposed to 1 mM dithiothreitol in 50 mM sodium glycerol phosphate–1 mM EDTA buffer (pH 6.8) at 23° for the times indicated. For experimental details, see Methods.

the peaks were sufficiently resolved could be taken between 30 and 50 min after addition of dithiothreitol. At this time the monomer peak had disappeared and the dimeric structure was grossly reestablished. It is difficult to decide on the basis of these observations whether the reappearance of the original (dimeric) structure coincides in time with restoration of activity, because full activity was certainly not yet recovered at the time the dimeric structure was re-formed (*cf.* Table II). One might conclude because of the time lag between recovery of activity and complete release of the blocking groups (compare Table II and Figure 4) that the monomers *b per se* have little or no activity. However, a slow reshuffling process leading to an active conformation of the monomers followed by rapid association of the monomers to dimers is not excluded.

Dimer b. The results in Table II indicate that phosphorylase *b* had retained some activity following DTNB treatment.[5] In Table I it was shown that the residual activity after DTNB treatment resides predominantly in dimer *b*. This made it possible to study the allosteric properties of the DTNB derivative of phosphorylase *b* and of the dimer *b* fraction isolated by sucrose density gradient centrifugation. Use was made of an earlier observation (Helmreich and Cori, 1964a) that a concave upward curve is obtained in double-reciprocal plots when the activity response of phosphorylase *b* to 5′-AMP is measured. This kinetic behavior is an expression of homotropic cooperativity between the binding sites of 5′-AMP in phosphorylase *b* (see also Madsen and Shechosky, 1967). Homotropic cooperativity between 5′-AMP sites in phosphorylase *b* was

[4] A considerable increase in specific activity was noted (up to 60 μmoles of product formed/mg of enzyme per min) when denatured protein was removed by centrifugation.

[5] A derivative of phosphorylase *b* with five mixed disulfides per mole of dimer *b* is inactive. This inactive preparation cannot bind 5′-AMP to a measurable extent (K_{diss} = >5 mM).

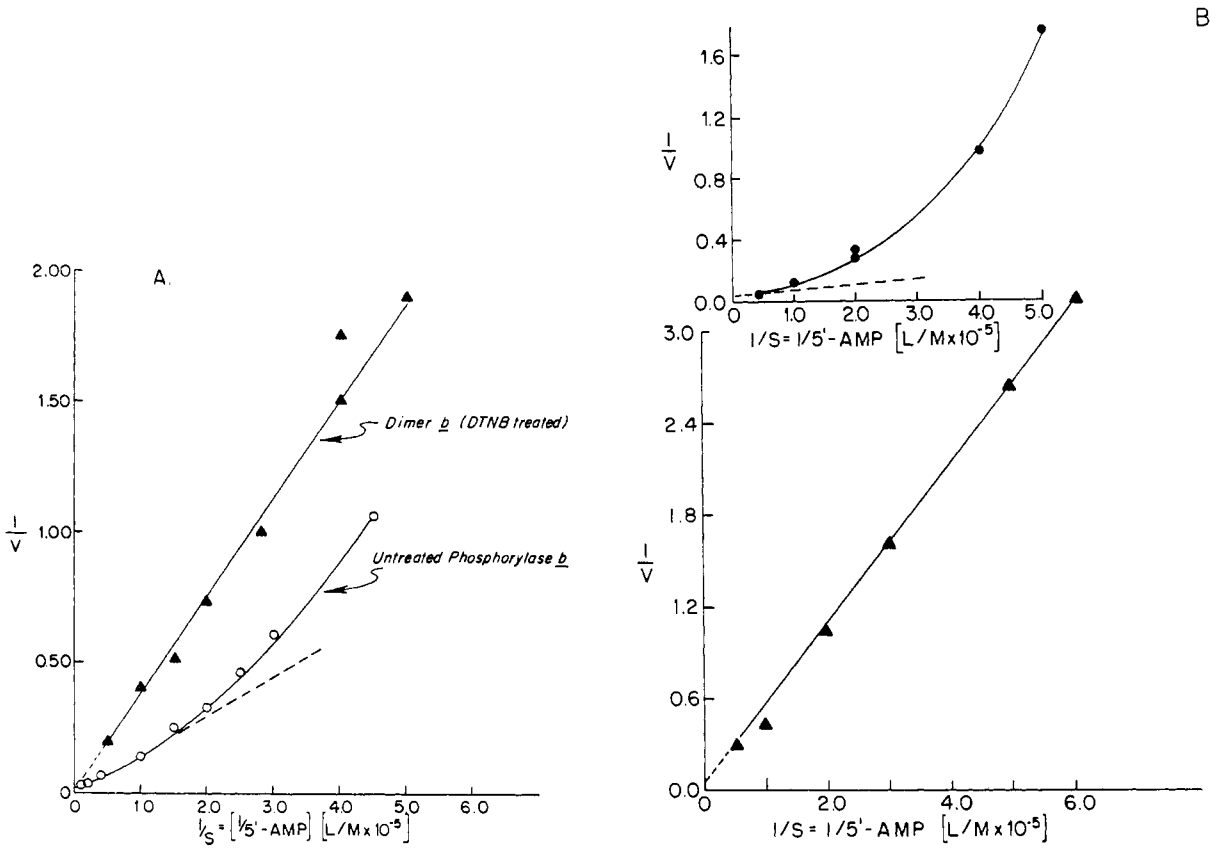

FIGURE 5: Double-reciprocal plots for the activation of DTNB-treated phosphorylase *b* by 5′-AMP. In panel A are shown data for the dimer *b* fraction (—▲—▲—) obtained by sucrose density gradient centrifugation from a phosphorylase *b* preparation containing four nitrophenylate groups in mixed disulfide linkages per mole of dimer *b* (see Figure 2). Untreated phosphorylase *b* (—○—○—) serves as control. In panel B are shown data for an unfractionated phosphorylase *b* preparation also containing four nitrophenylate groups per mole of dimer *b*. (—▲—▲—); no dithiothreitol was added. The data shown in the insert represent the same enzyme preparation (—●—●—●—) which was now incubated for 6 hr at room temperature with 1 mM dithiothreitol prior to rate measurements. The unusually large rate observed in the absence of 5′-AMP was deducted (see text). The buffer used was 8 mM sodium glycerol phosphate–3 mM EDTA buffer (pH 6.8), and the assay temperature was 23°. The concentrations of glycogen and glucose-1-P were 0.6 % and 50 mM, respectively. In the control incubations with native phosphorylase *b*, in panel A, 1.5 mM 2-mercaptoethanol was also added. In the incubations with the dithiothreitol-reactivated DTNB derivative of phosphorylase *b*, 1 mM dithiothreitol was also present during rate measurements. The concentration of enzyme was 25 μg/ml in each case.

also demonstrated recently in binding experiments (Kastenschmidt *et al.*, 1967; L. L. Kastenschmidt, J. Kastenschmidt, and E. Helmreich, unpublished data; Avramovic and Madsen, 1968). The kinetic test system was used in the experiments shown in Figure 5. It can be seen from Figure 5A,B that the homotropic cooperativity between the modifier sites is abolished after DTNB treatment in the dimer *b* fraction and in unfractionated phosphorylase *b*. In both instances the lines in double-reciprocal plots are linear. The lower curve in Figure 5A shows the behavior of native phosphorylase *b*. This curve has a concave upward curvature. The Hill coefficient is 1.5 in the range of 5′-AMP concentrations from 2×10^{-6} to 1×10^{-5} M. There is a considerable increase in the concentration of 5′-AMP required for half-maximal activation in the case of the DTNB derivatives of dimer *b* and phosphorylase *b* (see Figure 5A,B). The values are 2.0×10^{-4} and 1.4×10^{-4} M for the former and the latter preparation, respectively, but 7.2×10^{-5} M for the native enzyme. The value for the untreated enzyme was estimated from a Hill plot (*cf.* Methods, see also Helmreich *et al.*, 1967). In the case of the dimer *b* fraction shown in Figure 5A the change in the affinity of the enzyme for 5′-AMP following DTNB treatment occurs without change in V_{max}. Hence phosphorylase dimer *b* treated with DTNB, no longer exhibits cooperative site–site interactions, although its catalytic properties remain intact. We can conclude that reaction of two (certainly no more than three) SH groups per dimer *b* with DTNB weakens the interaction between the two monomeric subunits and leads to normal (hyperbolic) kinetics with respect to the activation of the dimer *b* with the modifier, 5′-AMP. Dimer *b* does not dissociate into two monomers under these conditions.

Several attempts were made to study the time course of reappearance of allosteric kinetic behavior. The DTNB derivatives of phosphorylase *b* were exposed to dithiothreitol from 16 min up to 6 hr prior to rate measurements which were carried out in the presence of 1 mM dithiothreitol (see insert in Figure 5B). The results suggest that complete homotropic cooperativity

is regained only after incubation with dithiothreitol for several hours (as is the case in the recovery of full activity; see Table II). An experiment showing sigmoidal activity response of DTNB-treated phosphorylase *b* to 5′-AMP after incubation with dithiothreitol for 6 hr is shown in the insert to Figure 5B. Hill's interaction coefficient was 2.0 in the range of 5′-AMP concentrations from 2 to 5×10^{-6} M. The concentration of 5′-AMP required for half-maximal activation was also estimated graphically from a Hill plot. It was 5×10^{-5} M. Both values agree with those for native phosphorylase *b* (see Figure 5A). However these enzyme preparations under the conditions of the assay had in the absence of 5′-AMP considerably more activity than native phosphorylase *b*. Their specific activity measured without 5′-AMP was 2.75 in the case of reactivated phosphorylase *b* but was only about 0.5 (or less) μmole of product formed/min per mg of enzyme in the case of native phosphorylase *b*. Accordingly there was a significant change in the activity ratios −5′-AMP/+5′-AMP. For example, in the case of the DTNB-treated and dithiothreitol-reactivated enzyme, shown in the insert of Figure 5B, this ratio (× 100) was 5.7 (using V_{max} at saturating (infinite) concentration of 5′-AMP), but was only about 1% in the case of native phosphorylase *b*. Since a measurable rate in the absence of 5′-AMP was observed with each of the dithiothreitol-reactivated DTNB derivatives of phosphorylase *b*, the rate in the absence of 5′-AMP was deducted from the rate observed in the presence of 5′-AMP in order to obtain the curve shown in the insert to Figure 5B.

These findings indicate that phosphorylase *b* even after removal of the thiol-blocking group by dithiothreitol did not fully regain its sensitivity toward the modifier. It may be noted from inspection of the curves for the DTNB enzyme derivatives in Figure 5A,B that the activity of these preparations is low at the smallest concentration of 5′-AMP tested. Therefore these preparations have prior to reduction with dithiothreitol an activity ratio, −5′-AMP/+5′-AMP, which is smaller or equally as small as that of native phosphorylase *b*.

These observations and the long delay in the recovery of allosteric and catalytic functions after removal of the blocking groups by dithiothreitol raise the possibility of disulfide interchange. This could involve the same SH groups in the protein that are reactive with DTNB.

Disulfide-interchange reactions might preclude the correct alignment of subunits immediately following the release of the blocking groups. The problem here is that one does not really know what the exact steps and what aside from the primary sequence the determining factors are in the formation of quarternary structures. But be that as it may, the evidence indicates that the allosteric properties of phosphorylase *b* are more sensitive to the action of thiol-reactive groups than the catalytic function. This makes it possible to destroy allosteric regulation without affecting catalytic activity. However, when the subunits in phosphorylase *b* are uncoupled the enzyme loses affinity for the modifier 5′-AMP. This might mean that now all of the enzyme is present in the T state, but other possibilities are not excluded.[6]

In the following section we wish to present data which illustrate the situation in reverse. It was found that preparations of apophosphorylase *b* reconstituted with pyridoxal or 5′-deoxypyridoxal bind 5′-AMP strongly and in a highly cooperative manner. Moreover these preparations show heterotropic cooperativity since their affinity for 5′-AMP is increased or decreased on addition of the substrate glucose-1-P or of the negative modifier glucose-6-P. However, these preparations are completely devoid of enzymatic activity as shown first by Illingworth *et al.* (1958) and subsequently by Shaltiel *et al.* (1966) and Hedrick *et al.* (1966).[7]

Allosteric Properties of Apophosphorylase b and of $NaBH_4$-Reduced Phosphorylase b. The apoprotein *b* has a somewhat lower binding affinity for 5′-AMP. The values for K_{dissn} of 5′-AMP are 5.3 and 3.7×10^{-4} M for the apo- and the holoenzyme, respectively, as determined under comparable conditions in binding experiments at 23° (*cf.* Tables III and V). Borohydride-reduced phosphorylase *b* has similar binding properties as native untreated phosphorylase *b* (Table III). It retained about 70% of the enzymatic activity before reduction and showed heterotropic cooperativity, *i.e.*, an increase in binding affinity for 5′-AMP on addition of glucose-1-P. These results were expected because Fischer *et al.* (1963) had already reported that the concentrations of 5′-AMP required for half-maximal activation of the $NaBH_4$-treated enzyme and of native phosphorylase *b* are similar. Furthermore Sealock and Graves (1967) reported that reduced phosphorylase *b* exhibits the same sigmoidal activity response to 5′-AMP as the unreduced enzyme. This has now been confirmed by binding measurements.

It may be noted that addition of glucose-1-P fails to increase the binding of 5′-AMP to apophosphorylase *b*. This is in contrast to the response of the holoenzyme to glucose-1-P (see Tables III and IV). (For additional data, see Kastenschmidt *et al.*, 1967; Helmreich, 1967; L. L. Kastenschmidt, J. Kastenschmidt, and E. Helmreich, unpublished data.) Moreover under certain conditions glucose-1-P also increases the binding of 5′-AMP to the 5′-deoxypyridoxal analog of phosphorylase *b*. This is shown in Table IV. The possibility that glucose-1-P is not effective because it cannot bind to apophosphorylase *b* was considered. However, high concentrations of glucose-1-P (100 mM) were also completely ineffective (Table III). The increase in the K_{dissn} values for 5′-AMP in these experi-

[6] A similar increase in the requirement for 5′-AMP for activity was noted by Jōkay *et al.* (1965) on treatment of phosphorylase *b* with PMB. Therefore there may exist a relationship of certain SH groups to the binding site for 5′-AMP on phosphorylase *b*. However what is not clear is whether this relationship is steric or allosteric. The structural relationships of the 5′-AMP binding sites and the SH groups in phosphorylase need to be studied.

[7] We have confirmed these observations. No activity was found in these preparations when they were tested in the direction of glycogen breakdown with P_i or of synthesis with glucose-1-P.

TABLE IV: Binding of 5′-AMP to Pyridoxal and to 5′-Deoxypyridoxal Phosphorylase *b*.[a]

Additions (mM)	In Glycerol-P Buffer[b]		In Glycylglycine Buffer	
	Pyridoxal Phosphorylase *b*, K_{dissn} (M × 10^4)	5′-Deoxypyridoxal Phosphorylase *b*, K_{dissn} (M × 10^4)	Phosphorylase *b*, K_{dissn} (M × 10^4)	5′-Deoxypyridoxal Phosphorylase *b*, K_{dissn} (M × 10^4)
None	0.53	0.53	>50	>50
Glucose-1-P (50)	0.60	0.55		
Glucose-1-P (20)			0.8	4.6
Glucose-6-P (50)		>50.0		

[a] Two different preparations of phosphorylase *b* reconstituted with 5′-deoxypyridoxal were used for the binding experiments in sodium glycerol-P and in glycylglycine buffer, respectively. All experiments were carried out at 23° and the composition of the sodium glycerol-P buffer, the pH and ionic strength (Γ/2 = 0.13), and the concentration of 5′-AMP were the same as in the experiments in Table III. The concentration of pyridoxal, of 5′-deoxypyridoxal phosphorylase *b*, and of phosphorylase *b* was 8.4 mg/ml in each case. The glycylglycine buffer contained 50 mM glycylglycine, 2 mM EDTA, and 1 mM 2-mercaptoethanol (pH 7.2) and Γ/2 was 0.13. The entries give the results of two experiments. [b] Control experiments carried out under identical conditions with phosphorylase *b* are given in Table III.

ments is due to the increase in ionic strength from Γ/2 = 0.13 to 0.47 resulting from the addition of a high concentration of anions (see Helmreich *et al.*, 1967). Therefore an effect of ionic strength is still discernible, although the specific allosteric ligand (glucose-1-P) is not able to induce the formation of a tighter binding form of the apoprotein in both the low and the high ionic strength buffer solutions. The contention that the absence of heterotropic cooperation between substrate and modifier binding sites is a characteristic property of the apoprotein is further supported by the data shown in the insert to Figure 6. The Hill coefficient for apophosphorylase *b* and 5′-AMP is 1.2 in the range of 5′-AMP concentrations from 9.4 × 10^{-5} to 2.2 × 10^{-4} M, where interaction is maximal. Although this indicates that some homotropic interactions between the binding sites for 5′-AMP may still occur, the cooperativity is much weaker in the apoenzyme as compared with the deoxypyridoxal derivative of phosphorylase *b* or the holoenzyme (*cf.* Figure 6 and subsequent text). The interaction coefficient for 5′-AMP and holophosphorylase *b* is 2.0 under comparable experimental conditions and for 5′-AMP concentrations from 4 to 10 × 10^{-5} M. Additional data will be presented; (L. L. Kastenschmidt, J. Kastenschmidt, and E. Helmreich, unpublished data). The experiments with apophosphorylase *b* in the insert of Figure 6 were carried out at 28° and in the absence of glucose-1-P. The K_{dissn} value for 5′-AMP and apophosphorylase *b* is about 7.0 × 10^{-4} M (see Table V). In view of the molecular heterogeneity and polydispersity of the apophosphorylase protein, it would not be surprising if some residual interactions between subunits should still occur. These interactions, however, must be very weak since the optical rotatory dispersion measurements of Hedrick (1966) showed a complete lack of response of apophosphorylase *b* to 5′-AMP. Heterogeneity of apophosphorylase protein was first noted by Illingworth *et al.* (1958) and more recently studied by Hedrick *et al.* (1966). The ultracentrifugal sedimentation velocity pattern of apophosphorylase *b* at 25° indicated a fast-sedimenting peak with an $s_{20,w}$ value of about 15.9 S which comprised 65% of the total area for the sedimenting material and a minor slow-sedimenting peak which

TABLE V: Effect of Temperature on the Sedimentation Behavior and the Affinity of Apophosphorylase *b* for 5′-AMP.[a]

Temp (°C)	$s_{20,w}$ × 10^{13} (S)	% of Total	K_{dissn} 5′-AMP (M × 10^5)
28			70[b]
25	5.2[c]	35	67
	7.4	65	
	15.9		
20			13
16	7.3	35	3
	13.6	65	
13			29
10	7.8	34.5	19
	15.1	65.5	

[a] Binding experiments and sedimentation velocity measurements were carried out in 50 mM sodium glycerol-P, 1 mM EDTA, and 2 mM 2-mercaptoethanol buffer, Γ/2 = 0.13 (pH 6.8) at the temperatures indicated. With the exceptions noted (experiments from Figures 6 and 7), the protein concentration was 12 mg/ml and the concentration of 5′-AMP was 3 × 10^{-4} M. The average of two experiments is given. [b] The K_{dissn} value was obtained from the experiments shown in the insert in Figure 6. [c] This experiment is shown in Figure 7.

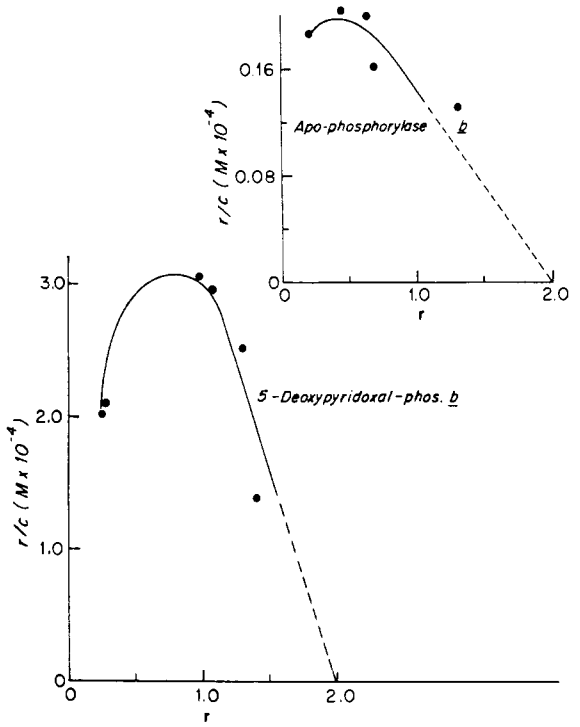

FIGURE 6: Binding of 5′-AMP to apophosphorylase *b* and to the phosphorylase *b* protein reconstituted with 5′-deoxypyridoxal. The binding experiments were carried out as described in Methods. Here is also given the mode of calculation for binding experiments. The experiments with apophosphorylase *b* shown in the insert were done at 28°. The concentration of protein was 11 mg/ml. The composition of the sodium glycerol phosphate buffer (pH 6.8), $\Gamma/2$ = 0.13, is described in Methods. The experiments with 5′-deoxypyridoxal phosphorylase *b* were carried out at 23°. The concentration of protein was 9.3 mg/ml.

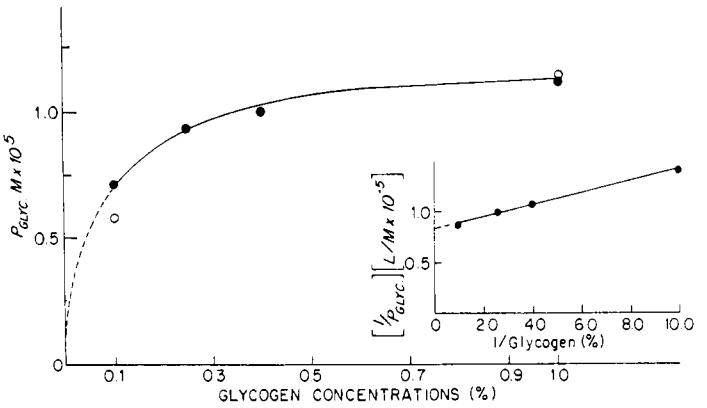

FIGURE 7: Binding of glycogen to apophosphorylase *b*. The binding experiments were carried out in 25 mM sodium glycerol phosphate, 1 mM EDTA, and 0.5 mM 2-mercaptoethanol (pH 6.8) at 20°. Data for apophosphorylase *b* (—●—●—) and, for comparison, a few data obtained with holophosphorylase *b* (—○—○—) are shown. The concentration of protein was in each case 2.36 mg/ml. On the ordinate is plotted the concentration of the protein–glycogen complex (P_{Glyc}) in M × 10^5 protein or the reciprocal of it (see insert). The concentration of glycogen is given in per cent (0.2 % corresponds to 1 × 10^{-3} M concentrations in terms of glucosyl groups at the nonreducing end of the chains).

comprised the remaining 35% and probably constitutes a mixture of monomers ($s_{20,w}$ = 5.2 S) and dimers ($s_{20,w}$ = 7.4 S) (see Table V). The $s_{20,w}$ value of 15.9 S for the fast-sedimenting form is considerably larger than the $s_{20,w}$ value of tetramer *b* (13.0 S; *cf.* Kent *et al.*, 1958). It appears that the molecular species which constitute apophosphorylase *b* are quite similar to the molecular forms which constitute the DTNB-treated phosphorylase *b* (*cf.* Figure 3). The photograph is not shown here because similar photographs were previously reported (*cf.* Hedrick *et al.*, 1966).

In order to see whether the absence of heterotropic cooperativity in apophosphorylase *b* is not merely a consequence of greatly reduced binding affinity of the apoprotein for specific ligands, the binding of glycogen to apophosphorylase *b* was studied. There is little doubt that apophosphorylase *b* binds as tightly to glycogen as the holoenzyme (see Figure 7). From the double-reciprocal plot (insert Figure 7) one calculates a value for K_{dissn} of glycogen and apophosphorylase *b* of 0.067% which corresponds to 3.35 × 10^{-4} M in terms of end groups of glycogen. Madsen and Cori (1958) give a value for K_{dissn} of glycogen and phosphorylase *a* of 0.27% which corresponds to 1.35 × 10^{-3} M in terms of glucosyl end groups. A recalculation of the data of Madsen and Cori (1958) which takes the K_{equil} = (dimer)²/(tetramer) of phosphorylase *a* into account yields a value of 2 × 10^{-4} M in terms of glycogen end groups (Metzger *et al.*, 1967). Therefore the K_{dissn} values for glycogen are quite similar for apo- and holophosphorylase *b* and for phosphorylase *a*. Considering that addition of 5′-AMP decreases the K_m value for glycogen and phosphorylase *b*, there is also an agreement between the K_m value and the K_{dissn} value for glycogen and apo- and holophosphorylase *b*. In the presence of 1 × 10^{-2} M P_i and 3 × 10^{-5} M 5′-AMP, a K_m value of 1.4 × 10^{-4} M in terms of glycogen end groups was determined with phosphorylase *b* (Helmreich and Cori, 1964a).

These results are quite consistent with the general idea that apophosphorylase still binds to stereospecific ligands, but in order to see whether this also applies specifically to anionic substrates such as glucose-1-P, it will be necessary to carry out direct binding measurements with glucose-1-P similar to those with glycogen. Such experiments are planned.

Allosteric Properties of Apophosphorylase b, Reconstituted with Pyridoxal or 5′-Deoxypyridoxal. Combination of apophosphorylase *b* with 5′-deoxypyridoxal restores homotropic interactions between the nucleotide binding sites (Figure 6). Hill's interaction coefficient is 2.0 for 5′-AMP concentrations between 1 and 3 × 10^{-5} M. Extrapolation of the linear portion of the binding curve to an intercept on the abscissa yields a rough estimate of the number of 5′-AMP binding sites of apophosphorylase *b* and of the 5′-deoxypyridoxal derivative. This approximation suggests that all the phosphorylase proteins studied, apophosphorylase *b*, the 5′-deoxypyridoxal derivative, and holophosphorylase *b*, have the same number of binding sites (*i.e.*, *n* = 2) for 5′-AMP/185,000 daltons of dimer *b*. This confirms the binding stoichiometry of DeVincenzi

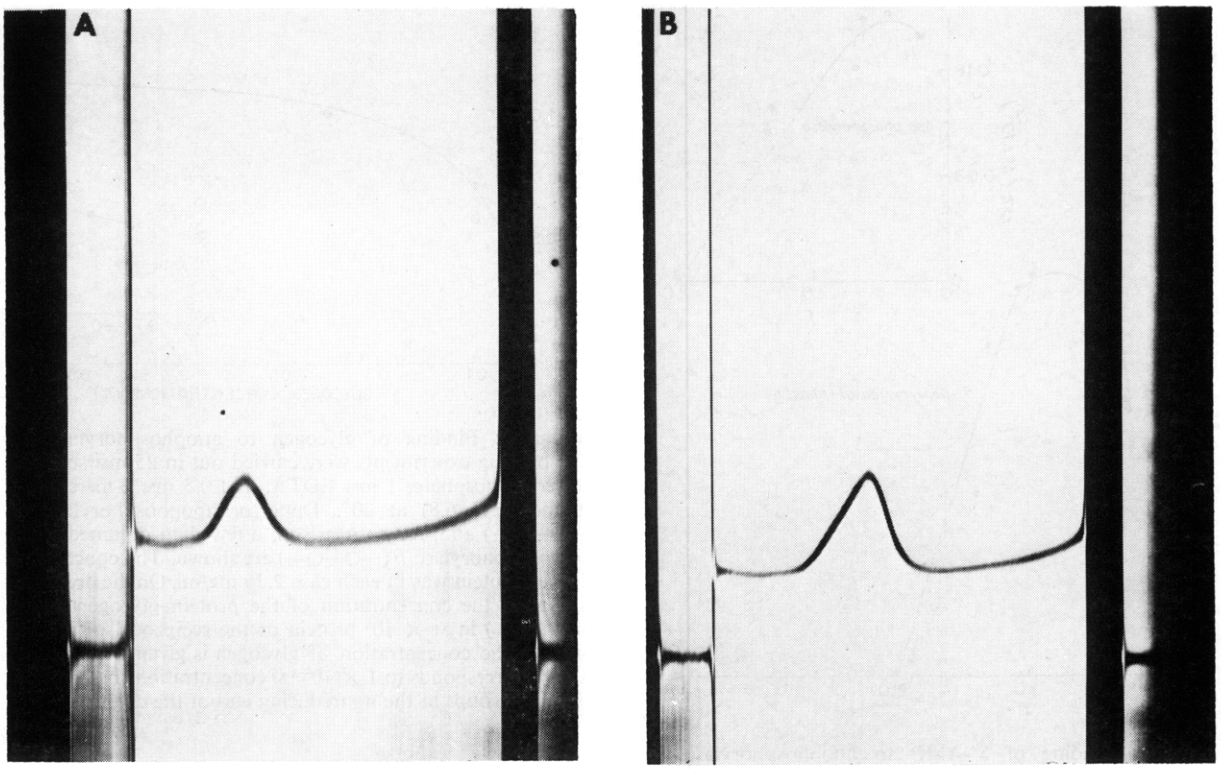

FIGURE 8: Sedimentation properties of phosphorylase *b* protein reconstituted with 5-deoxypyridoxal or pyridoxal at 35°. In panel A the 5′-deoxypyridoxal derivative of phosphorylase *b* is shown. A protein concentration of 6.6 mg/ml and 50 mM sodium glycerol-P, 2 mM EDTA, and 1 mM 2-mercaptoethanol buffer (pH 6.8) was used. The picture shown was taken 18 min after the rotor had attained full speed. In panel B the pyridoxal derivative of phosphorylase *b* is shown. The same buffer and temperature were used. The protein concentration was 3.56 mg/ml. The picture which is shown was taken 14 min after the rotor had attained full speed. Sedimentation in A and B was from left to right.

and Hedrick (1967). Additional data for phosphorylase *b* will be presented in a subsequent paper (L. L. Kastenschmidt, J. Kastenschmidt, and E. Helmreich, unpublished data).

Compared with the normal enzyme, the derivatives of phosphorylase *b* containing either pyridoxal or 5′-deoxypyridoxal bind 5′-AMP considerably tighter (see Table IV). For example, the K_{dissn} value for 5′-AMP and the 5′-deoxypyridoxal derivative of phosphorylase *b* in the particular experiment shown in Figure 6 is 3×10^{-5} M. Judging from their binding properties with respect to 5′-AMP, one could classify these analogs of phosphorylase *b* as R or tight-binding forms. If this is indeed the case and the T $\rightleftharpoons$ R equilibrium is shifted far to the right, an allosteric ligand such as glucose-1-P which is bound preferentially rather than exclusively to the R state would not be expected to have much effect in shifting further the equilibrium toward the R form. This would explain why glucose-1-P ($K_{dissn}(R)/K_{dissn}(T) = 0.08$ in glycerol-P buffer (pH 6.8); L. L. Kastenschmidt, J. Kastenschmidt, and E. Helmreich, unpublished data) fails to increase the binding of 5′-AMP to the derivatives of phosphorylase *b* but does so in the case of the active enzyme (Tables III and IV). Conversely, one should expect that a negative modifier, such as glucose-6-P (Morgan and Parmeggiani, 1964) which is bound preferentially to the T form, would shift the allosteric equilibrium of the 5′-deoxypyridoxal derivative of phosphorylase *b* toward the form (T) which has a low binding affinity for 5′-AMP.[8] Both predictions are borne out by the results in Tables III and IV. Subsequently (L. L. Kastenschmidt, J. Kastenschmidt, and E. Helmreich, unpublished data) we will show that replacing the glycerol phosphate anion by the glycylglycine cation in the buffer solutions markedly decreases the binding affinity of phosphorylase *b* for 5′-AMP. In glycylglycine buffer the allosteric equilibrium is shifted far to the left and phosphorylase *b* exists mainly in the T state. Use was made of this observation here and the effect of glucose-1-P on the binding of 5′-AMP to the 5′-deoxypyridoxal derivative of phosphorylase *b* was studied in glycylglycine buffer. It was anticipated that glucose-1-P, a positive modifier, would shift the equilibrium to the R state in glycylglycine buffer. This prediction also was proved to be correct (see Table IV). Therefore heterotropic cooperativity is indeed expressed in the 5′-deoxypyridoxal derivative of phosphorylase *b*, although compared with active phosphorylase *b* glucose-1-P is not quite as effective as allosteric ligand. (This suggests that the ratio $K_{dissn}(R)/K_{dissn}(T)$ for glucose-1-P and 5′-deoxypyridoxal phosphorylase *b* in glycylglycine buffer is >0.08.)

[8] Effects of glucose-6-P on the binding of 5′-AMP to holophosphorylase *b* are discussed in a later paper (L. L. Kastenschmidt, J. Kastenschmidt, and E. Helmreich, unpublished data).

We conclude that on substitution of pyridoxal 5-phosphate by 5′-deoxypyridoxal (or pyridoxal) a phosphorylase *b* protein is formed which has a molecular arrangement that allows for normal subunit interactions. This protein has regained, at least in a qualitative sense, allosteric properties characteristic for the active enzyme containing pyridoxal 5-phosphate. As expected, the sedimentation velocity patterns of 5′-deoxypyridoxal (or pyridoxal) phosphorylase *b* resemble those of active phosphorylase *b* and differ from those of apophosphorylase *b* (*cf.* Table V and Figures 8 and 10). This was noted first by Illingworth *et al.* (1958). In Figure 8 are shown ultracentrifugal sedimentation patterns for the 5′-deoxypyridoxal- and pyridoxal-reconstituted phosphorylases *b*. The experiments were carried out under conditions (35° and in the absence of 5′-AMP) which favor dissociation of the apoprotein (see Table V). However only single peaks with $s_{20,w}$ values of about 9 S and neither monomers nor aggregates were observed.

Effects of Temperature on Quarternary Structure and Binding Affinity of Apo-, Holo-, and 5′-Deoxypyridoxal-Phosphorylase b for 5′-AMP. Recently, Hedrick *et al.* (1966) have studied in detail the quarternary structure of apophosphorylase *b* under a variety of conditions including changes in protein and 5′-AMP concentrations, in temperature, pH, and ionic strength. We have now added studies of the binding properties of apo-, holo-, and 5′-deoxypyridoxal phosphorylase *b* with respect to 5′-AMP at different temperatures and pH and have attempted to correlate binding properties with quarternary structure. The binding measurements of the apophosphorylase *b* and the holoenzyme were carried out under conditions which are comparable (with respect to protein concentration, buffer ions, ionic strength, pH, addition of thiol compounds, and concentration of 5′-AMP) with the conditions of the sedimentation velocity studies reported in the literature (Kent *et al.*, 1958; Graves *et al.*, 1965; Hedrick *et al.*, 1966; Chignell *et al.*, 1968) and of additional studies with phosphorylase *b* (L. L. Kastenschmidt, J. Kastenschmidt, and E. Helmreich, unpublished data). Therefore only a few data of our ultracentrifugal studies with apophosphorylase *b* are reported here mainly to show that our data agree in general with those already published (see Table V). In addition we include a few data that are not available in the literature. These are sedimentation patterns for the 5′-deoxypyridoxal derivative of phosphorylase *b* at 35 and 13° and for phosphorylase *b* at 13° at pH 6.8 and 8.0 (see Figures 8 and 10). The conditions closely resemble those of the binding experiments in Figure 9.

Apophosphorylase *b*, at the temperatures studied, 10–25°, and in the presence of 3×10^{-4} M 5′-AMP, represents an associating–dissociating system. At the higher temperatures (25°) the protein is dissociated to a considerable extent (35%) into monomers and dimers. The remainder of the apoprotein is present in aggregated form ($s_{20,w}$ = >tetramer *b* = 15.9 S; see Table V). This is in contrast to phosphorylase *b* and its 5′-deoxypyridoxal and pyridoxal derivatives. These proteins do not aggregate at these temperatures nor

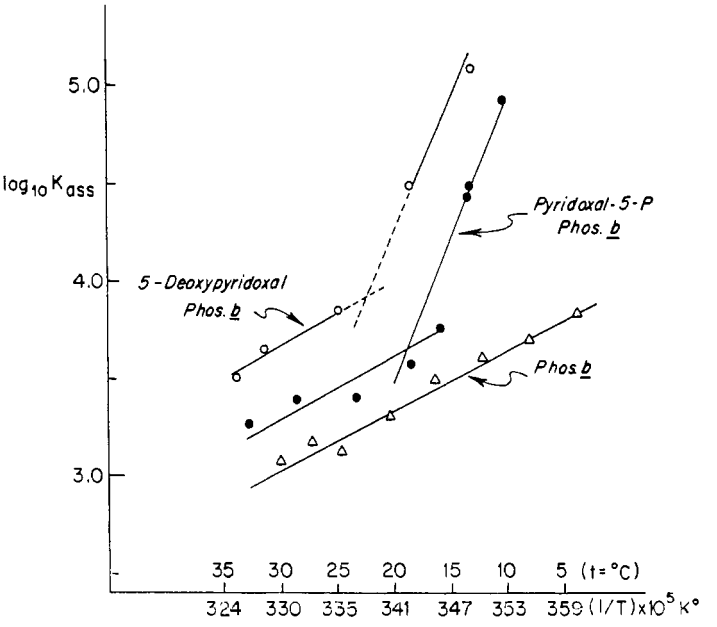

FIGURE 9: The effect of temperature on the binding of 5′-AMP to holophosphorylase *b* and to the 5′-deoxypyridoxal derivative of phosphorylase *b*. The experimental technique, the calculations, and the sodium glycerol-P buffer used in binding experiments are described in Methods. The concentrations of phosphorylase *b* and of the 5′-deoxypyridoxal derivative were 16.1 and 18 mg per ml, respectively. Only one concentration (3×10^{-4} M) of 5′-AMP was used, but several points represent an average of two experiments. (○—○—○) 5′-Deoxypyridoxal derivative (pH 6.8), (●—●—●) phosphorylase *b* (pH 6.8), and (△—△—△) phosphorylase *b* (pH 8.0).

do they dissociate into monomers. They exist as dimers (*cf.* Figure 8 and Table V). In the low-temperature range all the phosphorylase *b* proteins studied had a tendency to associate. However, there is one significant difference with respect to the quarternary structure of apophosphorylase *b* and the holoenzyme or its 5′-deoxypyridoxal derivative. Whereas apophosphorylase *b* at pH 6.8 and at 10° forms aggregates of a molecular weight > tetramer *b*, the holoenzyme and the 5′-deoxypyridoxal-reconstituted proteins form rather typical tetramers at pH 6.8 and at 13°. Data for apophosphorylase *b* are given in Table V and data for phosphorylase *b* and 5′-deoxypyridoxal phosphorylase *b* in Figure 10. The $s_{20,w}$ values for the apoprotein are 7.8 and 15.1 S. The values for phosphorylase *b* under comparable conditions are 11.8 (91%) and 8.2 S (9%) and for the 5′-deoxypyridoxal derivative 12.6 S. 5′-AMP further promotes association at low temperatures (see Hedrick *et al.*, 1966) and lowering the pH has a similar effect. Graves *et al.* (1965) could show, for example, that even native phosphorylase *b* can form aggregates of higher molecular weight when incubated for 2 hr at 0° in sodium glycerol-P buffer at pH 6.0. These aggregates are similar to those formed by the apoprotein. Under these conditions they found two boundaries on ultracentrifugation with $s_{20,w}$ values of 8.3 and 20.2 S.

It is tempting to correlate the rather sudden change in ligand affinity (see Figure 9) with subunit association.

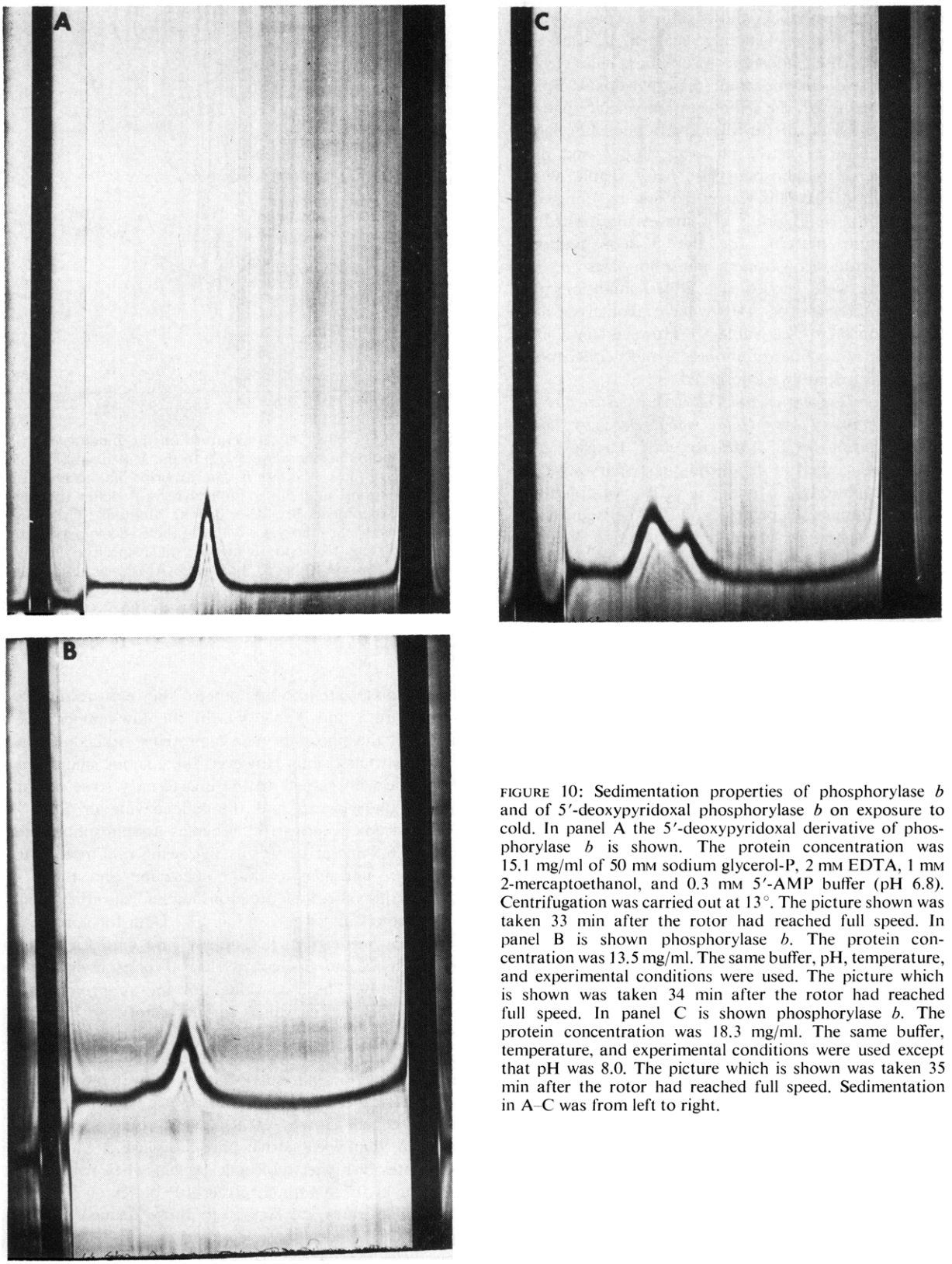

FIGURE 10: Sedimentation properties of phosphorylase *b* and of 5′-deoxypyridoxal phosphorylase *b* on exposure to cold. In panel A the 5′-deoxypyridoxal derivative of phosphorylase *b* is shown. The protein concentration was 15.1 mg/ml of 50 mM sodium glycerol-P, 2 mM EDTA, 1 mM 2-mercaptoethanol, and 0.3 mM 5′-AMP buffer (pH 6.8). Centrifugation was carried out at 13°. The picture shown was taken 33 min after the rotor had reached full speed. In panel B is shown phosphorylase *b*. The protein concentration was 13.5 mg/ml. The same buffer, pH, temperature, and experimental conditions were used. The picture which is shown was taken 34 min after the rotor had reached full speed. In panel C is shown phosphorylase *b*. The protein concentration was 18.3 mg/ml. The same buffer, temperature, and experimental conditions were used except that pH was 8.0. The picture which is shown was taken 35 min after the rotor had reached full speed. Sedimentation in A–C was from left to right.

It will be noted from Table V that in the case of apophosphorylase *b* maximum binding affinity actually coincides with a quarternary structure (dimers and tetramers) resembling that of native phosphorylase *b*. Therefore in the case of the apophosphorylase *b* protein a correlation does exist between a decrease in binding affinity (both for the higher and the lower range of temperatures) and an altered quarternary structure (see Table V).[9] Active phosphorylase *b* and 5′-deoxypyridoxal phosphorylase *b* preparations have a lesser tendency to associate (*cf.* Table V and Figure 10). It may be noted that in the case of the apoprotein the binding affinity for 5′-AMP actually decreased below 16° (see Table V), whereas it increased in the case of phosphorylase *b* and the 5′-deoxypyridoxal analog (Figure 9). Around 22–18° there is a sharp break in the van't Hoff plots and the slopes rise steeply when the temperature gradually falls to 13 and 10°. At the higher temperatures (from the transition point up to 33°), the enthalpy change, $-\Delta H$, is 12.7 kcal, then from the transition temperature on downward to 10° the slopes rise sharply and the enthalpy change with respect to the binding of 5′-AMP becomes 61.8 kcal/mole. It should be noted that at the higher temperatures the slope for the apoprotein and the slopes for the holoenzyme and the 5′-deoxypyridoxal derivative of phosphorylase *b* have the same degree of rise. The inflection in the curves also occurs for all three phosphorylase proteins in about the same range of temperature.

We conclude that subunit interactions in the active phosphorylase *b* protein and in the 5′-deoxypyridoxal derivative of phosphorylase *b* are similar as judged from the effect of temperature on quaternary structure and binding properties, but that subunit interactions in the apoprotein are different. One would like to know what contact regions and physicochemical forces within the monomers are responsible for the association of the phosphorylase proteins on exposure to cold. This requires a different kind of experimentation.

Graves *et al.* (1965) found that phosphorylase *b* at pH 6.0 lost activity rather sharply at temperatures below 13°. This phenomenon was ascribed to cold inactivation of phosphorylase *b*. Because of the lability of the pyridoxal–protein-bonded structure to low pH (*cf.* Shaltiel *et al.*, 1966), the experiment of Graves *et al.* (1965) was repeated at pH 6.8 by Dr. B. E. Metzger. Under our conditions, the break in the Arrhenius plot occurs in the same temperature range as the break in the van't Hoff plots (compare Figures 9 and 11). For other examples of discontinuities in Arrhenius plots, see Massey *et al.* (1966). As expected from the original observation of Graves *et al.* (1965), the higher pH not only shifted the transition temperature from 13 to 18°, it also decreased the extent of cold inactivation. This is evident from a comparison of the enthalpy changes ($-\Delta H$) at pH 6.0 and 6.8. Graves *et al.* (1965) obtained for the lower temperatures (4.6–13°) a value for $\Delta H = -46$ kcal/mole and for the higher temperatures (13–30°) a value of -17 kcal. This may be compared with Figure 11, where one obtains for the lower temperatures (10–18°) a value for $\Delta H = -22.6$ kcal/mole and for the higher temperatures (18–33°) a value of -16 kcal.

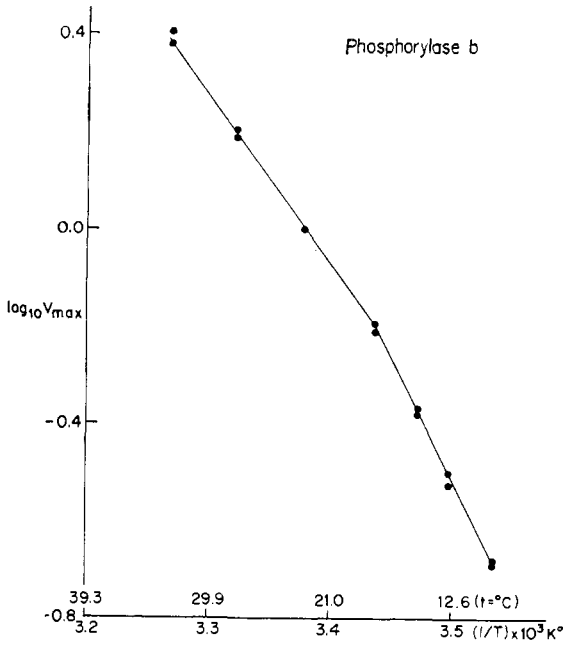

FIGURE 11: Arrhenius plot of phosphorylase *b* activity. Log V_{max} is plotted against $1/T$°K. Activity measurements involved arsenolysis of glycogen. The concentrations of 5′-AMP, glycogen, and arsenate were 1 mM, 0.5%, and 0.2 M, respectively. pH was 6.8. The conditions were those described by Helmreich and Cori (1964b) (see also Metzger *et al.*, 1967, 1968).

These observations led us to study the binding of 5′-AMP to phosphorylase *b* at pH 8.0. These experiments are included in Figure 9. It can be seen that only one slope (without break) is obtained over the whole range of temperatures (4–29°). The slope again is the same as that obtained with the apo-, holo-, and 5′-deoxypyridoxal phosphorylase proteins at the higher temperatures up to the inflection point. If one now correlates these results with the quarternary structure of phosphorylase *b* at pH 8.0 (see Figure 10), it becomes clear that subunit interactions seem indeed to be responsible for the unusual effect of temperature on the binding properties of the phosphorylase protein as was first suggested by Graves *et al.* (1965). At pH 8.0 phosphorylase *b* exists predominantly in the form of dimer *b* (see Figure 10) (61% has a $s_{20,w}$ value of 8.3 S and 39% is a tetramer with a $s_{20,w}$ value of 11.5 S).

The experiments in Figure 11 represent measurements of V_{max} at saturating concentrations of substrates (including glycogen) and of 5′-AMP. Phosphorylase *b* is present as dimer *b* under these conditions (*cf.* Wang *et al.*, 1965; Metzger *et al.*, 1967, 1968). It thus follows that there also exists a correlation between the conformational rearrangement within the mono-

[9] The binding affinity of the apoprotein for 5′-AMP in the temperature range between 16 and 20° is about equal to the binding affinity of the holoenzyme. Judging from the binding affinity for 5′-AMP one would conclude that the subunits in the apophosphorylase protein are intact and not unfolded.

meric units in dimer *b* upon exposure to cold and a decrease in activity. On the basis of these correlations we would like to conclude that although differences exist (notably between apo- and holophosphorylase *b*) weakening of subunit interactions brought about by such different means as exposure to cold, blocking SH groups, or removal of pyridoxal 5-phosphate, to name only a few conditions, is responsible for a general tendency of the subunits to associate and for changes in binding properties, catalytic activity, and regulatory functions.

Discussion

The information obtained in this work bears on the nature of contact between subunits of phosphorylase *b*. The evidence points to one (or two) SH groups per monomer because formation of mixed disulfides between these SH groups and thionitrophenylate groups results in dissociation of dimers *b* to monomers *b*. Thus, these particular SH groups seem to be directly or indirectly involved in the contact between the two monomers in the dimer *b*.[10] Our data which indicate differential reactivity of SH groups in the monomers, in the dimers, and in the aggregated material (see Figure 2, Table I) are somewhat reminiscent of the all or none effect of PMB on the dissociation of rabbit skeletal muscle phosphorylase *a* described by Madsen and Cori (1956). These authors and Madsen and Gurd (1956) found that the monomers *a* were completely saturated with PMB, assuming that 18 moles of SH groups/mole of (original) phosphorylase tetramer *a* are reactive with PMB, whereas the tetramer *a* had only one or two of its SH groups reacted with PMB, and had lost little or no activity. A comparison of the present results with the earlier experiments of Madsen and Cori (1956) and of Madsen and Gurd (1956) points to the advantage of the less reactive and more discriminating Ellman's reagent over the organomercurial. With this information now available a chemical attack on the peptide sequences containing these SH groups seems feasible. This should give more direct information on the chemical nature of the contact regions between subunits in phosphorylase *b*.

Additional physicochemical information is also required with respect to the forces which hold the subunits together in phosphorylase. The rather sudden changes in binding affinity, catalytic activity, and quaternary structure of muscle phosphorylase proteins in a critical temperature range could provide a clue. At least in the case of the holoenzyme and the 5′-deoxypyridoxal analog of phosphorylase *b* the increase in binding affinity for 5′-AMP and the trend toward subunit association with a decrease in temperature and pH would seem to argue against the involvement of hydrophobic regions. The involvement of charged groups and of electrostatic interactions seems more likely and would be in line with a drastic change in the temperature dependency of 5′-AMP binding to phosphorylase *b* at pH 8.0 as compared with pH 6.8. But before the actual chemical groups that are responsible for subunit contact in phosphorylase are known, speculation is not profitable.

However, one can say that the allosteric and the catalytic functions of this enzyme can be differentiated by various means as shown in this work. Allosteric and catalytic functions of phosphorylase have also different requirements with respect to the chemical structure of the prosthetic group. For example the inactive 5′-deoxypyridoxal analog of phosphorylase *b* and the active holoenzyme undergo similar changes in binding properties and quarternary structure with changes in temperature. Thus, combining the apoprotein with 5′-deoxypyridoxal results in subunit interactions which are quite similar to those of the holoenzyme. However this derivative differs from phosphorylase *b* in one important aspect. It is devoid of activity. Although the presence of the phosphate group in pyridoxal 5-phosphate (or for that matter of phosphorylserine groups as in the case of phosphorylase *a*) has an effect on subunit contact and quarternary structure, as one might expect, these effects of charged groups on the structure of the protein may represent rather general electrostatic effects. We have now found that B_6 analogs which combine with apophosphorylase *b* through their 4-carbonyl group but lack the 5-phosphate group can restore the allosteric properties (and the quarternary structure) of the native enzyme. It is quite possible that contact between subunits in the apophosphorylase protein can be reestablished by much less specific means than by binding of pyridoxal 5-phosphate or of vitamin B_6 analogs lacking the 5-phosphate group. Indeed Fischer and Krebs (1966) state that apophosphorylase proteins can form aggregates when combined with carbonyl groups totally unrelated to the 4-carbonylpyridoxal structure. However quarternary structure and allosteric properties have not yet been reported for these aggregates. Thus considering the wide variety and diversity of reagents which are known to affect subunit contact in oligomeric proteins in general (Schachman, 1963) and in phosphorylase specifically (see Sealock and Graves, 1967), the possibility becomes less attractive that the only function of pyridoxal 5′-phosphate in phosphorylase should be that of a conformational determinant. If one wishes to consider an additional role for the prosthetic group of phosphorylase, attention obviously centers on the 5′-phosphate group of pyridoxal 5-phosphate, since the phosphate group is apparently essential for catalytic activity, whereas its role with respect to the allosteric properties of the enzyme protein is permissive as shown in this paper. Since pyridoxal 5-phosphate does not donate its phosphate group directly in the form of a reactant as shown by Illingworth *et al.* (1958), one might consider a possible involvement of the phosphate group of pyridoxal 5-phosphate similar to that of the carboxyl group in aspartate 52 in lysozyme (Philipps, 1967). This would imply a stabilizing influence of the phosphate anion of the pyridoxal 5-

[10] After this manuscript was completed Dr. Neil B. Madsen kindly informed us of a paper by Batell *et al.* (1968). These authors present data on the reactivity of SH groups of phosphorylase *b* with DTNB that are in agreement with the data presented here.

phosphate group on an intermediate carbonium ion (C_1) which is bonded to a glycosidic oxygen. However, before speculation becomes more profitable the structural relationships between the pyridoxal 5-phosphate site and its environment in the protein need to be clarified.

Acknowledgments

We wish to thank Miss Carmelita Lowry for helping us with the ultracentrifugal analyses and Mr. Roger W. Sherman for assistance with the experiments. We are indebted to Dr. Boyd E. Metzger for the experiments shown in Figure 11. We are obligated to Drs. Henri Buc and Neil B. Madsen for making information available to us prior to publication.

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Substrate Synergism and Phosphoenzyme Formation in Catalysis by Succinyl Coenzyme A Synthetase*

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ABSTRACT: The phosphorylated form of succinyl coenzyme A synthetase (E-P) is shown by rapid mixing and quenching experiments to participate on the major or exclusive catalytic pathway. The initial rate of appearance of E-P is as least as great as the initial steady-state rate of the over-all reaction in either direction and, in the direction of adenosine triphosphate synthesis, the steady-state level of E-P appears to be reached before the establishment of the steady-state rate of adenosine triphosphate formation. Succinyl coenzyme A synthetase provides an excellent example of a phenomenon termed substrate synergism. This refers to acceleration by a substrate or substrates of a reaction undergone by other substrates of a multisubstrate enzyme. Theoretical considerations show that such synergism can be conclusively detected by appropriate comparison of rates of isotopic exchange between certain substrates in the presence or absence of other substrates. Substrate synergism occurs with succinyl coenzyme A synthetase as measured by the adenosine diphosphate $\rightleftarrows$ adenosine triphosphate, succinate $\rightleftarrows$ succinyl coenzyme A, and E-P $\rightleftarrows$ adenosine triphosphate exchange reactions. Acceleration of the adenosine diphosphate $\rightleftarrows$ adenosine triphosphate exchange by succinyl coenzyme A or by all other substrates likely reflects modifications important in net catalysis because both the exchange and over-all reaction probably involve E-P as an intermediate.

Multisubstrate enzymes frequently catalyze partial reactions that may reflect steps in the over-all catalysis. For example, succinyl-CoA[1] synthetase will catalyze an ADP $\rightleftarrows$ ATP exchange (Kaufman, 1955), and aminoacyl-tRNA synthetases will catalyze a P–P_i $\rightleftarrows$ ATP exchange (DeMoss and Novelli, 1956). With succinyl-CoA synthetase, as with other enzymes, such partial reactions have frequently been observed to be relatively slow, thus raising doubts as to whether the reactions responsible are participants in the catalysis. A plausible explanation often considered is that the presence of other substrates may markedly increase the rate of the step responsible for the partial reaction. Such rate acceleration could logically arise from existence of a synergism between or among substrates for promotion of catalysis. Evidence for such acceleration has been presented with succinyl-CoA synthetase (Ramaley *et al.*, 1967), aminoacyl-tRNA synthetases (Hele, 1964; Ravel *et al.*, 1965; Mitra and Mehler, 1966; Deutscher, 1967; Lee *et al.*, 1967), and argininosuccinate synthetase (Rochovansky and Ratner, 1967). A definitive synergism is shown in the promotion of acylation of glyceraldehyde 3-phosphate dehydrogenase by the binding of DPN (see Malhotra and Bernhard, 1968). With citrate synthase, L-malate, probably by mimicing oxalacetate, promotes exchange of the methyl hydrogens of acetyl-CoA (Eggerer, 1965). This likely represents acceleration of a catalytic step. Closely related to synergism among substrates are observations of McElroy *et al.* (1967) and of Baldwin and Berg (1966), demonstrating promotion of hydrolysis of enzyme-bound intermediates by presence of other substrates.

Convincing experimental and theoretical means for quantitative demonstration of such substrate synergism have been lacking. One purpose of this paper is to present data obtained with succinyl-CoA synthetase, together with theoretical considerations, that clearly establish occurrence of substrate synergism in this enzymic catalysis.

The findings on substrate synergism are intimately

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: succinyl-CoA, succinyl coenzyme A; acetyl-CoA, acetyl coenzyme A.