Role of Pyridoxal 5′-Phosphate in Glycogen Phosphorylase. III. Assembly of the Pyridoxal 5′-Phosphate Site in Rabbit Skeletal Muscle Phosphorylase *b* Studied by Tritium–Hydrogen Exchange†

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ABSTRACT: The hydrogen–tritium exchange of phosphorylase *b* and phosphorylase *b* derivatives which were labeled *prior*, *during*, or *after* liganding of the apoenzyme with pyridoxal-P, pyridoxal-P analogs, or 5′-AMP has been examined. Gradually prolonged exposure of apophosphorylase to [^{3}H]H_2O prior to reconstitution classified the afterward retarded exchange rates of more than 400 hydrogens. The hydrogens "trapped" in the course of reconstitution with the cofactor indicated the formation of a hydrophobic pocket from previously exposed or more motile regions of the protein. The number of trapped hydrogens was proportional to the ratio of phosphorylase *b* bound pyridoxal-P or analogs. If pyridoxal-P was added to apophosphorylase aggregated in the presence of 5′-AMP, the trapping was decreased to approximately half the number of hydrogens. Some new qualitative and quantitative conclusions can be drawn from these results on hydrogen exchange. Pyridoxal-P is a major conformational determinant which affects the arrangement or motility of at least one-quarter of phosphorylase *b*. Phosphorylase *b* derivatives reconstituted with analogs of pyridoxal-P are clearly distinguishable by different exchange rates and trapping effects. The 3′-*O*-methylpyridoxal-P exhibits less catalytic activity because it cannot apparently stabilize the active conformation to the same extent. Specifically, the cofactor binding site becomes more solvent accessible. Reconstitution of apophosphorylase *b* by 3′-*O*-methylpyridoxal-P appears to be a biphasic process in which a rapid binding and induced conformational change was followed by a slow regain of enzymatic activity. Enzymatically inactive pyridoxal did not affect hydrogen exchange of the phosphorylase proteins in a manner comparable to pyridoxal-P. However pyridoxal-P monomethyl ester, reconstituted a phosphorylase *b* preparation which showed remarkable similarities in its hydrogen-exchange kinetics to active phosphorylases. This inactive analog appears to fulfill the steric requirements for the assembly of the cofactor binding site like active pyridoxal-P analogs. The influence of 5′-AMP on the exchange reaction reveals a partial overlap of the contact surfaces responsible for formation of holophosphorylase dimers and 5′-AMP-dependent aggregates of apophosphorylase *b*. Comparison of the results obtained with oligomeric phosphorylase *b* and monomeric *Escherichia coli* D-serine dehydratase allowed further to distinguish between tertiary and quarternary structure changes during the reconstitution process.

Pyridoxal-P[1] and 5′-AMP profoundly affect the tertiary and quarternary structure of muscle glycogen phosphorylase *b*. These differences are most clearly seen in a comparison of the dimeric holoenzyme and the monomeric apoenzyme and the events leading to resolution of the holoenzyme or reconstitution of the apoenzyme (Fischer and Krebs, 1966; Shaltiel *et al.*, 1966; Helmreich, 1969; Fischer *et al.*, 1970). Studies of gross conformational changes which alter the association–dissociation equilibria of oligomeric phosphorylase were mainly carried out with ultracentrifugal techniques (Hedrick *et al.*, 1966; Kastenschmidt *et al.*, 1968a,b) and by gel electrophoresis (Hedrick *et al.*, 1969; Chignell *et al.*, 1968). More localized or small structural changes could be detected by differences in the reactivity of SH groups which result from exposure or protection of SH groups in the various forms of the enzyme (Avramovic-Zikic *et al.*, 1970). Recently spectroscopic probes were used as sensitive tests to minor structural changes induced by 5′-AMP on holophosphorylase *b* (Birkett *et al.*, 1971). However, these probes cannot quantitatively describe the extent of the structural changes in the assembly of the pyridoxal-P site.

Recent investigations of conformational changes by hydrogen exchange could demonstrate the sensitivity of this method to detect structural rearrangements in the whole protein. Different conformations of the same protein may be distinguished by the number and rate of exchangeable hydrogens (Hvidt and Nielsen, 1966). Ligand binding to the protein or structural rearrangements which move exposed regions of the protein to the interior will affect the back-exchange of previously labeled hydrogens. If ligand binding decreases the "motility" of the protein, the retarded back-exchange can easily be measured by the number of "trapped" hydrogens (Schechter *et al.*, 1968, 1969; Budzynski and Fraenkel-Conrat, 1970). Reversible transitions between different conformations could be shown to cause retardation *or* acceleration of hydrogen exchange (Ulmer and Kaegi, 1968). In an attempt to refine the exchange conditions we could show, that *short time* "exchange-in" is especially suited to assess qualitatively and quantitatively the rearrangement of defined portions of the protein.

Despite the similarity of the hydrogen exchange method with other procedures for the analysis of group reactivity,

† From the Department of Physiological Chemistry, University of Würzburg, Würzburg, Germany. *Received December 21, 1971.* A preliminary report of a part of this work was given at the Joint Biochemical Symposium USSR-GDR, May 3–7, 1971, Schloss Reinhardsbrunn. For previous publications in this series, see Pfeuffer *et al.* (1972a,b). Supported by grants of the Deutsche Forschungsgemeinschaft, The Federal Ministry of Education and Science and the Fonds der Chemie.

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[1] Abbreviations of vitamin B_6 analogs follow IUPAC–IUB rules (1970). In figures and tables the trivial names were replaced by the following abbreviations: PL is pyridoxal and PLP is pyridoxal 5′-phosphate; glycogen phosphorylase (EC 2.4.1.1): α-1,4-glucan:orthophosphate glucosyltranferase.

"hydrogen trapping" has the advantage to avoid permanent alterations in the protein. Therefore we have studied by hydrogen exchange the reconstitution of apophosphorylase *b* with pyridoxal-P and pyridoxal-P analogs and the influence of 5′-AMP on various stages of this process.

Materials and Methods

Materials. Tritiated water (200–5000 mCi/g) and [1-^{14}C]-acetic anhydride (25–60 mCi/mmole) were obtained from The Radiochemical Centre, Amersham. The sources of the other reagents were as follows: polyethylene glycol with an average molecular weight of 17,000–20,000 (Serva, Heidelberg), Sephadex G-25 fine (Pharmacia GmbH), pyridoxal and pyridoxal 5′-phosphate·H_2O (Merck, Darmstadt). 3′-*O*-Methylpyridoxal-P, 3′-*O*-methylpyridoxal-P *N*-oxide, pyridoxal-P monomethyl ester, and pyridoxal-P *N*-oxide were kindly provided by Dr. T. Pfeuffer (Pfeuffer *et al.*, 1972a).

Polyethylene Glycol [1′-^{14}C]Diacetate. The [^{14}C]acetic anhydride (0.3 mCi) was contained in a "break-seal" ampoule. It was quantitatively transferred and diluted to 0.1 mequiv with unlabeled acetic anhydride in 290 μl of absolute benzene by distillation in a closed system. The break-seal tube was then replaced by another reaction flask, containing 1.58 g of polyethylene glycol (0.158 mequiv), 47.4 ml of absolute benzene, 63 μl of pyridine, and 1 mg of 4-dimethylaminopyridine (Steglich and Höfle, 1969), and combined with the labeled acetic anhydride. The mixture was refluxed in a closed system for 3 hr at 60–70° and finally reacted with a 10% molar excess of cold acetic anhydride. A concentrated aqueous solution of the ether precipitated reaction product was placed on a Sephadex G-25 column (1.2 × 30 cm). The homogeneous fraction (70% of total) which emerged first from the column was collected and freeze-dried. The product was diluted with unlabeled polyethylene glycol to the desired concentration. Usually polyethylene glycol [^{14}C]acetate with a specific activity of 40,000 dpm/mg was used. [1-^{14}C]Acetylchloride can be used instead of [^{14}C]acetic anhydride for the preparation of polyethylene glycol [^{14}C]acetate with equally good results.

D-*Serine dehydratase* from *E. coli*[2] and the PLP-free apoenzyme were prepared according to Dowhan and Snell (1970). Reconstitution of the D-serine dehydratase from apoenzyme and pyridoxal-P or pyridoxal-P analogs was carried out as described by the same authors.

Phosphorylase b was prepared from fresh rabbit skeletal muscle by the procedure of Fischer and Krebs (1958) and Krebs *et al.* (1964), recrystallized at least three times, and stored under toluene in the cold room. All the enzyme preparations were recrystallized once more prior to use. The specific activities of the phosphorylase *b* preparations ranged from 60 to 66 μmoles of $P_i \cdot min^{-1} \cdot mg^{-1}$ if assayed according to Kastenschmidt *et al.* (1968a). The measurement of protein concentration using $A_{280}^{1\%} = 13.2$; methods for the removal of 5′-AMP and the preparation of the apoenzyme are described in Pfeuffer *et al.* (1972a).

Reconstitution of apophosphorylase b with pyridoxal-P or analogs of pyridoxal-P was carried out as described by Hedrick *et al.* (1966). Pyridoxal-P or the analogs were allowed to react with the apoprotein at up to 100-fold molar ratio per binding site in 50 mM glycerol-P–50 mM 2-mercaptoethanol buffer (pH 7.0). Bound cofactor was assayed by the procedure of Wada and Snell (1961). For further details, see Pfeuffer *et al.* (1972a). All calculations are based on a molecular weight of 100,000 and one pyridoxal-P specific binding site per phosphorylase *b* monomer (Cohen *et al.*, 1971).

Hydrogen Exchange. The two-column separation technique of Englander and a ^{14}C-labeled marker as monitor of the enzyme concentration was used (Englander, 1963, 1968). To 0.3–0.5 ml (containing 1.5–5 mg of protein) an exactly measured amount of polyethylene glycol [^{14}C]acetate was added in glycerol-P buffer (pH 7.0). Tritiation was started by additions of [^{3}H]H_2O to a final concentration of 20 mCi/ml. Ligands which induce conformational changes were added *prior* to, *during*, or *after* tritiation (see Results). The "exchange-in" period was terminated by a rapid Sephadex column passage into label-free buffer or ligand-substituted buffers. All columns were of equal diameter (2 cm) and filled with 2.5 g (dry weight) of Sephadex G-25. The sample was placed on the gel surface and pumped through the gel at constant speed. In control experiments, the protein in the effluent was determined by continuous ultraviolet absorbancy measurements. The protein effluent was pooled and incubated for exchange-out at a concentration of 0.5–1.5 mg/ml. At the end of the exchange-out period, the protein was separated from the medium as described above, except that 0.2–0.4 ml of the solution (approximately 0.1–0.6 mg of protein) was removed at preset times. The protein effluent was collected and counted.

Radioactivity Assay. All samples were counted in 10 ml of Bray's solution (Bray, 1960) in a Tri-Carb Model 3380 liquid scintillation spectrometer. Calibration data from internal standards were used to calculate ^{3}H and ^{14}C disintegrations per minute by the external standard channel ratio procedure (Turner, 1971). Average counting efficiencies were 60% for ^{14}C and 26% for ^{3}H at double-isotope settings.

Calculation of Exchange Data. The formula given by Englander (1963) was used to calculate N, the number of labeled hydrogens per mole of protein. The formula can be adapted to ^{14}C radioactivity measurements using [^{14}C]-polyethylene glycol acetate to measure protein concentrations as follows: $N = 111(T/T_0) \times (C_0/C) \times M_w = 111\,(C_0/T_0) \times M_w \times R$, where C_0 is the ^{14}C disintegrations per minute of the labeled marker added per milligram of protein, C is the ^{14}C disintegrations per minute actually determined in any given aliquot of the sample, T_0 is the tritium counts (disintegrations per minute per milliliter) in the original mixture, T is the tritium counts in the same aliquot of the experimental sample, and 111 is the molar concentration of hydrogens in water. Thus, one only needs to determine $R = T/C$ to obtain N. The ratio R of a phosphorylase *b* control served additionally to standardize individual experimental series. If the molecular weight of the protein is unknown, one may use an approximate equivalent number of exchangeable hydrogens per mg of protein.

Kinetic Analysis of Exchange Data. The kinetics of hydrogen–protein exchange may be treated as a series of simultaneous first-order reactions (Hvidt and Nielsen, 1966)

$$H(t) = \sum_{i=1}^{m} n_i \cdot e^{-k_i t}$$

where $H(t)$ is the number of unexchanged hydrogens per mole at time t, n_i and k_i are the total number of hydrogens and the rate constant for the ith kinetic class, respectively, and m is the total number of kinetic classes. Despite the assumptions for the theoretical case, where m has a limiting value equal to the number of (identical) exchangeable sites, experimental

[2] We are greatly indebted to Professor E. E. Snell for making available to us a coli mutant (C6 of *E. coli* K12) which we have used as a source for the preparation of crystallized D-serine dehydratase.

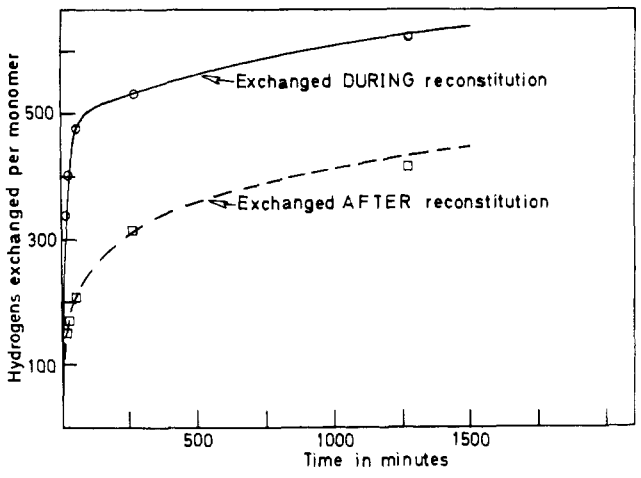

FIGURE 1: Time course of hydrogen–tritium exchange-in as a function of reconstitution of apophosphorylase *b*. Total exchange-in time is indicated in the abscissa. The upper curve was obtained by addition of PLP 20 min before termination of exchange-in. The lower curve was obtained from tritiation after completion of reconstitution. The experimental points are calculated from exchange-out data and do not consider immeasurably fast- exchanging hydrogens. For other conditions, see legend to Table I.

hydrogen exchange curves can be usually approximated by a small number of kinetic classes (Hvidt and Nielsen, 1966; Segal and Harrington, 1967). A more complete treatment of exchange kinetics on a statistical basis was given by Laiken and Printz (1970). We computer analyzed our preliminary data of more than 10 data–time points for each experiment and 7–10 duplicate experiments by the method of successive subtraction for the minimal number of rate constants which would fit the data within 10%. Average rate constants close to the preliminary estimates of several experiments were then used to fit the data to a multiple linear regression which generated n for each kinetic class. Hydrogens exchanging with half-times less than 2 min were not accounted for. Three widely spaced kinetic classes with half-times from 10 to 1000 min were found, a fourth class considers extremely slow exchanging hydrogens with half-times substantially higher than 1000 min. On the basis of this experimental background comparative studies with scarce analogs were carried out with 7 data–time points, adequately spaced to the range of the kinetic classes. All calculations were carried out with a Wang 720B/702 computer with digital plotter.

Results

Use of ^{14}C-labeled polyethylene glycol of sufficiently large molecular weight as an indicator of the protein in the column effluent was first recommended for hydrogen–protein exchange by Englander (1963). We prepared a labeled derivative of polyethylene glycol by end-group acetylation. The indicator molecule, when added to a phosphorylase *b* solution of known concentration, moved together with the protein on Sephadex G-25 columns as ascertained by independent protein determinations. The results agreed within ±2%. Addition of the indicator to phosphorylase *b* at ratios ranging from 10 to 0.6 (w/w) did not alter the exchange behavior, enzymatic activity, and stability of the phosphorylase protein. The number of labeled hydrogens per mole of protein can be calculated directly from the $^{3}H:^{14}C$ ratio in a small volume (one or more drops) of the column effluent. The use of polyethylene glycol dispenses with additional methods for protein determination, thus simplifying the procedure and increasing its accuracy. Furthermore it makes feasible partial automation of tritium–protein-exchange experiments.

Kinetics of Tritium–Hydrogen Exchange of Pyridoxal-P Reconstituted Phosphorylase b. One can calculate from the amino acid composition of phosphorylase *b* (Appleman *et al.*, 1963; Saari, 1970) that 821 exchangeable peptide bonds and 758 exchangeable amino acid side-chain hydrogens (total 1579) are available per monomer with a molecular weight of 100,000 (Cohen *et al.*, 1971). Among these large numbers of hydrogens, the majority of the extremely fast- ("instantaneously") exchanging hydrogens originating from random coil portions of the polypeptide backbone cannot be assessed by gel filtration techniques at pH 7.0 (Englander, 1967; Englander and Poulsen, 1969; Woodward and Rosenberg, 1970). The functional moiety of the protein may be considered on the other hand to be built into a stabilized slowly exchanging region of the protein. Exchange conditions which can be consistently analyzed kinetically for the transformation of formerly fast- to afterward slowly exchanging hydrogens would be best suited to show the formation of a stabilized protein conformation.

We have chosen to focus our interest on experimental conditions where the protein in the unliganded conformation has approached exchange equilibrium only with the instantaneously and some fast exchanging hydrogens but where the remainder of the molecule was not yet labeled. This has the advantage that any alteration of the protein leading to a retardation of back-exchange, or conformation changes converting fast-exchanging regions of the protein to newly assembled slowly exchanging regions can be easily identified after the unchanged hydrogens have back-exchanged to essentially zero. Figure 1 illustrates the time dependent exchange-in of holophosphorylase *b* (lower curve) and the exchange-in of apophosphorylase *b*, which was reconstituted by addition of pyridoxal-P during tritiation (upper curve). After exchange-in intervals of 1, 5, 35, 245, and 1180 min (allowing 20 min to complete reconstitution) the back-exchange was started. The data are analyzed in Table I, using the smallest number of distinguishable rate classes to accommodate all exchange experiments (see Methods). The distinction is however still somewhat arbitrary, as pointed out by Laiken and Printz (1970). Specifically, the back-exchange of the unmodified controls after short time exchange-in shows, that only 1 or 2 classes would sufficiently fit the data. For comparison of the data one should, however, consider in each case the apparent distribution among all classes. In this case, a small number of hydrogens in the classes with small rate constants reflect the short exchange-in interval of the unmodified control and labeled hydrogens in the modified protein can clearly be distinguished from the control by the number of hydrogens in the otherwise unoccupied classes.

Considering that during the exchange-in intervals the exchangeable hydrogens of phosphorylase *b* were only partially equilibrated we assumed that exchange-in is first order to calculate by extrapolation maximal numbers for the various rate classes. We estimated 205 hydrogens in class I ($t_{0.5}$ = 13.86 min), 125 hydrogens in class II ($t_{0.5}$ = 125 min), and 138 hydrogens in class III ($t_{0.5}$ = 1000 min). The extremely slow exchanging hydrogens, class IV, can be estimated from the 150 hydrogens which become buried during reconstitution. The number of instantaneously exchanging hydrogens ($t_{0.5}$ < 2 min) must be equal or higher than the 187–261 hydrogens which are trapped after short time exchange-in.

TABLE I: Effect of Reconstitution of Apophosphorylase *b* with PLP during Exposure to Tritiated Solvent as a Function of Exchange-in Time.[a]

Exchange-in Interval before Initiation of Reconstitution (min)	Av Half-Lives (min) and No.[d] (*n*) of Back-Exchanging Hydrogens per Phosphorylase Monomer *b*				
	Class I ($t_{0.5}$ = 13.86) *n*	Class II ($t_{0.5}$ = 125) *n*	Class III ($t_{0.5}$ = 1000) *n*	Class IV ($t_{0.5}$ > 1000) *n*	ΣI–IV *n*
1	183	56	67	32	338
Control[b]	141	2	7	1	151
Δ[c]	42	54	60	31	187
5	188	97	66	55	406
Control[b]	156	3	8	1	168
Δ[c]	32	94	58	54	238
35	192	102	95	89	478
Control[b]	173	27	7	2	209
Δ[c]	19	75	88	87	269
245	182	130	93	130	535
Control[b]	194	93	23	5	315
Δ[c]	−12	37	70	125	220
1180	236	98	120	150	604
Control[b]	204	121	79	8	412
Δ[c]	32	−23	41	142	192

[a] Exchange-in of apophosphorylase *b* (6–9 mg/ml) in 50 mM glycerol-P–50 mM 2-mercaptoethanol buffer (pH 7.0), 23°, was started by addition of [^{3}H]H_2O. After the times indicated 2 moles of PLP/monomer was added. Exchange-in was terminated by gel filtration after allowing 20 min for complete reconstitution. For back-exchange, see Methods. [b] The controls were fully reconstituted prior to exchange-in of the protein for the same length of time. [c] Calculated difference of labeled hydrogens in phosphorylase *b* reconstituted during or prior to exchange-in. [d] *n* is computer generated, only integral values are given.

This totals 887 accountable hydrogens for holophosphorylase *b*.

The total number of modifications of exchangeable sites in the course of the reconstitution of the apoenzyme was calculated from the difference of classes I–IV in the modified and unmodified protein, *i.e.*, 42, 94, 88, and 142 hydrogens. (Negative differences probably indicate further shifts in the exchange characteristics.) The short exchange-in times reveal transpositions of instantaneously exchanging sites to slowly exchanging sites. Increasing exchange-in times may be analyzed for transpositions of labeled hydrogens between classes I–III and IV. Thus, at least 238 formerly instantaneously exchanging hydrogens have to be included in the number of modified exchangeable sites.

The comparison indicates that during binding of pyridoxal-P to the apoenzyme approximately 50% of all measurably exchangeable sites or 25% of all potentially exchangeable sites have altered their ability to exchange with the solvent. We have tried to overcome the ambiguity to relate distinguishable rate classes to certain phases of the reconstitution process by carrying out similar reconstitution studies with pyridoxal-P analogs and in the presence of 5′-AMP. The experimental conditions were chosen analogous to the 5-min exchange-in experiments (Table I), which proved the most favorable conditions to differentiate between modified and unmodified phosphorylases by means of the trapping effect.

Reconstitution of Apophosphorylase b with Pyridoxal-P Analogs. The structural role of pyridoxal-P in muscle glycogen phosphorylase might be studied by a comparison of the exchange kinetics of phosphorylase *b* derivatives formed upon reconstitution with enzymatically active and inactive pyridoxal-P analogs. The conventional methods compare different but stable conformations under identical exchange conditions (Hvidt and Nielsen, 1966).

All phosphorylase *b* derivatives were stoichiometrically reconstituted from the same apophosphorylase *b* preparation *prior* to tritiation. Exchange-in was carried out for 900 min. While the total number of exchangeable hydrogens was approximately the same with the various phosphorylase preparations tested, some differences in the kinetic distribution of exchangeable sites become apparent if one considers classes II–IV. Apophosphorylase *b* released its labeled hydrogens faster than holophosphorylase *b* and some of the pyridoxal-P analog reconstituted phosphorylases. This points to a looser structure of apophosphorylase *b*. A similar conclusion was also reached by Hedrick *et al.* (1966) who observed small differences in the sedimentation coefficients of apo- and holophosphorylase *b*. The back-exchange of pyridoxal phosphorylase *b* and 3′-*O*-methylpyridoxal-P *N*-oxide phosphorylase *b* resembled apophosphorylase *b*. This suggests weaker interactions of these analogs with the enzyme, which is in agreement with the evidence presented by Pfeuffer *et al.* (1972b). Although differences in exchange kinetics between some other pyridoxal-P analog carrying phosphorylase *b* derivatives were reproducible, it was difficult to validate these differences statistically (*cf.* Englander, 1967). This approach was therefore abandoned and the "hydrogen-trapping" method was used. Figure 2 illustrates the difference in back-exchange of phosphorylase reconstituted from apophosphorylase with pyridoxal-P analogs *prior* or *during* short time exchange-in.

TABLE II: Effect of Reconstitution of Apophosphorylase *b* with PLP Analogs during Exposure to Tritiated Solvent.

PLP Analog	Ratio of Added PLP Analog per Monomer	Reconstitution		Classes and No. (*n*) of Back-Exchanging Hydrogens per Monomer of Phosphorylase *b* Derivatives				
		Before Tritiation[a]	During Tritiation[b]	Class I *n*	Class II *n*	Class III *n*	Class IV *n*	ΣI–IV *n*
PLP	2	−	+	188	97	66	55	406
		+	−	156	3	8	1	168
				Δ 32	94	58	54	238
PL	10	−	+	127	6	16	6	155
		+	−	108	12	14	5	139
				Δ 19	−6	2	1	16[c]
3′-*O*-Me-PLP	10	−	+	217	118	52	5	392
		+	−	158	3	6	0	167
				Δ 59	115	46	5	225
PLP monomethyl ester	10	−	+	220	69	52	33	374
		+	−	144	8	6	1	154
				Δ 76	61	46	32	215

[a] Reconstitution was initiated by addition of the PLP analog to apophosphorylase *b* 20 min prior to exchange-in. Exchange-in was terminated after 25 min by gel filtration. [b] Apophosphorylase was exposed 5 min to tritiated solvent, followed by addition of the PLP analog. Exchange-in was terminated by gel filtration after allowing 20 min for complete reconstitution. [c] The number of hydrogens increases to 27 if PL was added in a molar ratio of 100. For other conditions, see legend to Table I.

The time interval was sufficiently long for regain of full enzymatic activity when active pyridoxal-P analogs were used. Pyridoxal (added at molar ratios from 10 to 100) was stoichiometrically bound to apophosphorylase *b* (as shown in Table IV) but did not retard hydrogen back-exchange with similar characteristics as pyridoxal-P (Figure 2). Since it is known that pyridoxal-reconstituted phosphorylase *b* forms dimers (Kastenschmidt *et al.*, 1968a; Shaltiel *et al.*, 1969), the results suggest that only loose aggregates are formed which readily dissociate. In this case, a slight retardation of instantaneously exchanging hydrogens would have escaped detection. In contrast, the short time exchange kinetics of 3′-*O*-methylpyridoxal-P and pyridoxal-P monomethyl ester reconstituted enzymes had many features in common with pyridoxal-P reconstituted phosphorylase (Table II). The number of hydrogens trapped was 225 for 3′-*O*-methylpyridoxal-P- and 215 for pyridoxal-P monomethyl ester phosphorylase *b*. These numbers closely approach the 238 hydrogens trapped by pyridoxal-P under the same conditions. For comparison, pyridoxal trapped only 16–27 fast hydrogens. Apparently with the former analogs and in contrast to pyridoxal the same number of amino acids participate in the structural rearrangement. However, the numbers in the rate classes are differently distributed after the conformational rearrangement in the pyridoxal-P analog phosphorylases and indicate a different degree of final stabilization. The 3′-*O*-methylpyridoxal-P phosphorylase *b*, for example, lacks slowly exchanging hydrogens (class III and IV) after reconstitution, which indicates a less stable cofactor binding region. An alternate explanation would be an equilibrium between an enzymatically active (hard to exchange) conformation with one or more enzymatically inactive (easy to exchange) conformations. The former conformation might more exactly fulfill the steric requisites of the cofactor binding site and explain the partial enzymatic activity.

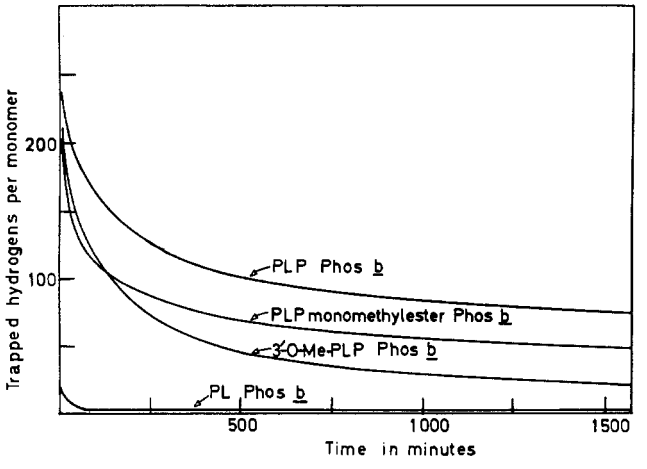

FIGURE 2: Trapping of labeled hydrogens during reconstitution of apophosphorylase *b* with PLP analogs. The number of labeled hydrogens from exchange-in during reconstitution is compared to the corresponding values of apophosphorylase *b*. The difference is plotted *vs.* time of exchange-out. For conditions, see Table III.

When the pyridoxal-P monomethyl ester is compared with pyridoxal-P a more similar distribution of trapped hydrogens in the classes III and IV is found. However, the pyridoxal-P monomethyl ester enzyme shows an increased portion of class I hydrogens, which, as will be discussed in more detail, might be related to a somewhat different quarternary structure.

Effect of 5′-AMP. Besides the nearly absolute dependence of phosphorylase *b* activity on 5′-AMP, several functional and conformational effects of the nucleotide are known. In holophosphorylase *b*, 5′-AMP favors association especially

TABLE III: Effect of 5′-AMP on Tritium–Hydrogen Exchange of Phosphorylase *b* Derivatives as a Function of the Sequence of Addition of the Ligands during Reconstitution.[a]

Expt	Cofactor Added[b]		Classes and No. (*n*) of Back-Exchanging Hydrogens per Monomer of Phosphorylase *b* Derivatives				
	Before Tritiation	During Tritiation	Class I *n*	Class II *n*	Class III *n*	Class IV *n*	ΣI–IV *n*
I	PLP		156	3	8	1	168
II		PLP	188	97	66	55	406
III	5′-AMP		181	5	0	1	187
IV		5′-AMP	260	27	5	1	293
V	5′-AMP	PLP	188	45	42	23	298
VI	PLP	5′-AMP	156	3	8	1	168
VII		5′-AMP + PLP	222	62	85	52	421
VIII	5′-AMP	3′-*O*-Me-PLP	204	50	20	4	278
IX		5′-AMP + 3′-*O*-Me-PLP	230	94	44	14	382

[a] Ligands were added to apophosphorylase *b* either 20 min before or 5 min after initiation of exchange-in. Tritiation was carried out for 25 min. [b] Molar ratios of added compound per monomer of apophosphorylase *b* (6–9 mg/ml) are PLP 2, 3′-*O*-Me-PLP 10, and 5′-AMP 3.1. For other conditions, see legend to Table I.

TABLE IV: Binding of Pyridoxal-P, Pyridoxal, and 3′-*O*-Methylpyridoxal-P to Apophosphorylase *b* as a Function of Time and Concentration.

Cofactor Added to Apophosphorylase *b*	Molar Ratio of Added Cofactor per Monomer	Protein (mg/ml)	Time (min)	Moles Bound/Monomer[a]
Holoenzyme–control		5.19		1.01 ± 0.03
Apoenzyme–control		5.19		0.1 ± 0.05
PLP	2.7	8.65	10	1.13 ± 0.06
PL	13.5	8.65	10	0.31 ± 0.10
			20	0.54 ± 0.06
			60	1.10 ± 0.05
3′-*O*-Me-PLP	13.5	8.65	10	0.85
			20	1.07
			60	1.08

[a] Assayed by the procedure of Wada and Snell (1961).

at low temperatures (see Kastenschmidt *et al.*, 1968b), and also protects certain SH groups against attack by SH reagents (Avramovic-Zikic *et al.*, 1970; Birkett *et al.*, 1971). Apophosphorylase *b* still binds 5′-AMP, but homo- and heterotropic cooperativity among effector and substrate binding sites is largely abolished (Kastenschmidt *et al.*, 1968a). In the presence of 5′-AMP, apophosphorylase *b* forms a sizeable amount of dimers (Hedrick *et al.*, 1966). We observed, in general agreement with these previous reports, that the exchange-out kinetics of apophosphorylase and reconstituted holophosphorylase *b* were affected differently by 5′-AMP. The allosteric modifier was added *before*, *together with*, and *after* addition of pyridoxal-P or its analogs (Table III, expt I–IX). The addition of 5′-AMP together with pyridoxal-P or after reconstitution (expt VII) had little or no additional effect on the exchange. But when 5′-AMP was added to the apoenzyme during tritiation, 106 hydrogens were trapped predominantly in classes I and II (expt IV–III). A plausible explanation would be that association of the monomeric subunits caused this retardation of back-exchange. This assumption supposes that the apoenzyme aggregates are itself in rapid equilibrium with their monomers. Whereas 238 hydrogens could be trapped by reconstitution of apophosphorylase *b* with pyridoxal-P, this number was reduced (108 less) if the 5′-AMP–apophosphorylase complex was reconstituted with pyridoxal-P under the same conditions (expt V–VI). Similar results were obtained with 3′-*O*-methylpyridoxal-P (expt VIII–VI). This number corresponds rather closely to the number of hydrogens which were already trapped during formation of the 5′-AMP–apophosphorylase complex.

A difference between the pyridoxal-P– and the less active 3′-*O*-methylpyridoxal-P–phosphorylase–AMP complex should be noted. In the case of the 3′-*O*-methylpyridoxal-P complex, fewer slowly exchanging hydrogens of the classes III and IV were trapped (expt IX). This again suggests a less rigid binding site in the case of 3′-*O*-methylpyridoxal-P

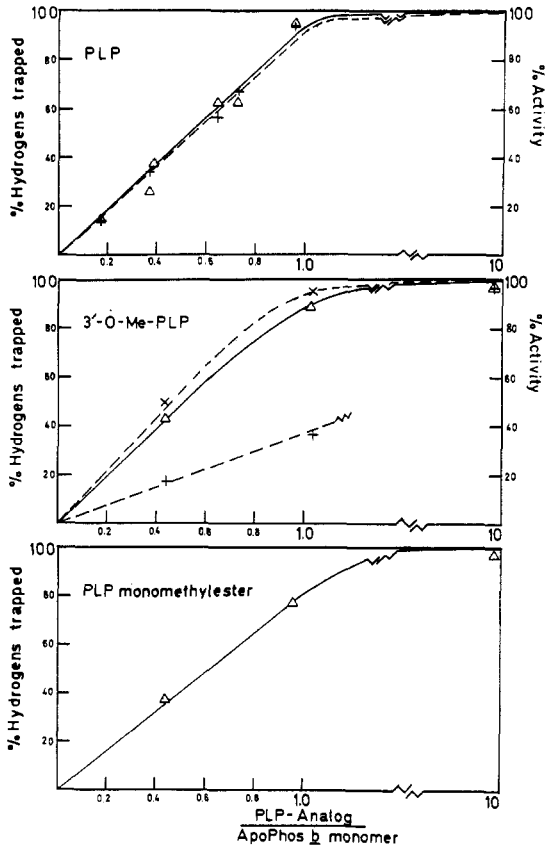

FIGURE 3: Reactivation and hydrogen trapping during reconstitution of apophosphorylase *b* as a function of the molar ratios of PLP or PLP analogs per binding site. (Δ) Tritium–hydrogen-exchange experiments were performed as outlined in Figure 2, the values correspond to samples taken 20 min after addition of the cofactor. Enzymatic activity was determined from samples taken 20 min (+) and 1200 min (×) after addition of the cofactor. 100% values are from experiments with a 2-fold (PLP) or 10-fold (3′-*O*-Me-PLP and PLP monomethyl ester) molar excess per binding site.

phosphorylase *b* and a relatively small influence of 5′-AMP on the stability of the cofactor binding region.

Partial Reconstitution of Apophosphorylase b. Hydrogen-exchange studies were carried out to correlate the extent of hydrogen trapping with the stoichiometry of cofactor binding and enzymatic reactivation during reconstitution. Pyridoxal-P and the analogs indicated in Table IV bind in a ratio of 2 moles of cofactor/dimer. However, even in presence of a large excess of pyridoxal or 3′-*O*-methylpyridoxal-P binding is slow.

If apophosphorylase was reconstituted with less than stoichiometric amounts of pyridoxal-P during short-time exchange-in also less labeled hydrogens were trapped. Figure 3 illustrates that a linear relationship exists between hydrogen trapping and reactivation. A lack of proportionality would have indicated the formation of hybrid molecules with different kinetics and/or specific activities.

As further demonstrated in Figure 3, the percentage of additionally trapped hydrogens also corresponds satisfactorily to the molar ratio of cofactor in the case of 3′-*O*-methylpyridoxal-P and pyridoxal-P monomethyl ester. An interesting fact is revealed by the comparison of reactivation and hydrogen trapping in the case of 3′-*O*-methylpyridoxal-P. At 23°, hydrogen trapping during binding of the ligand to apophosphorylase *b* is complete after 20 min, but maximal enzymatic reactivation is not yet attained. This indicates that the binding of the analog is not the rate-limiting step in the reactivation process, but binding is apparently followed by a slow rearrangement of the enzyme into a stable active conformation. This biphasic process explains differences with respect to the molar excess, time, and temperature required for reactivation of apophosphorylase *b* with 3′-*O*-methylpyridoxal-P (Shaltiel *et al.*, 1969; Pfeuffer *et al.*, 1972b). We made certain therefore that under the conditions of the hydrogen-trapping experiments (Tables II and III) reactivation was complete after 20 min at 23°.

Comparison to Other Enzymes. The apoenzyme of *Escherichia coli* D-serine dehydratase could be fully reactivated by stoichiometric amounts of pyridoxal-P at pH 7.5. Short time exchange-in during reconstitution (addition of pyridoxal-P 5 min after exposure to [^{3}H]H_2O, total tritiation time 25 min) leads to the following distribution of additionally labeled hydrogens per molecule as compared with a prior reconstituted control.

class I ($t_{0.5}$ = 10 min) 6	class II ($t_{0.5}$ = 100 min) 15
class III ($t_{0.5}$ = 1000 min) 31	class IV ($t_{0.5}$ > 1000 min) 7

The preincubation of serine apodehydratase with 5′-AMP did not affect hydrogen exchange.

Discussion

The recent progress in hydrogen-exchange methods provided a useful approach to study conformational changes of proteins by an evaluation of the number of labeled hydrogens which alter their ability to exchange during the structural rearrangements (Schechter *et al.*, 1969; Ulmer and Kaegi, 1968). A further refinement of this method is achieved, when the conformational change is induced before the slowly exchanging sites of the protein are equilibrated. This gives more confidence that the newly appearing slowly exchanging hydrogens originated from formerly instantaneously exchanging hydrogens and were immobilized during the conformational change.

Extended exchange-in of holophosphorylase gives an exchange profile, characterized by a number of distinguishable kinetic classes of fast- and slowly exchanging hydrogens. These classes, so far, cannot be directly related to other physical parameters, such as secondary, tertiary, or quarternary structure nor can they directly be assigned to any ligand binding site. However, the binding of a ligand in this case pyridoxal-P or 5′-AMP to a protein, *i.e.*, apophosphorylase, which was not yet completely equilibrated with respect to slowly exchanging hydrogens, may elicit to what extent the actual number of hydrogens in the slowly exchanging classes are dependent on the specific interaction of a given ligand with the protein.

Using this approach, we found that 400 exchangeable sites/monomer were altered in their exchange behavior by pyridoxal-P. From these hydrogens 187–269 originated from formerly instantaneously exchanging sites, probably at the solvent exposed surface of the apoenzyme. Their exchange rates were retarded by more than 1000-fold. This reflects a huge structural change and indicates that approximately 50% of the measurable exchanging sites or at least 25% of all potentially exchangeable sites in phosphorylase *b* are stabilized by the interaction of pyridoxal-P with the protein. Certainly, not all exchangeable sites, whose exchange is altered, are

necessarily a part of the cofactor binding site. It is known that quarternary structure changes accompany reconstitution.

A unique way to assess more specifically the mutual interdependence of the assembly of the pyridoxal-P site and of the quarternary structure was provided by D-serine dehydratase from *E. coli*. It is a monomeric pyridoxal-P-dependent enzyme with mol wt 45,000 (Dowhan and Snell, 1970). Reconstitution of D-serine apodehydratase with pyridoxal-P during tritiation traps 59 predominantly slow exchanging hydrogens. Considering the molecular weights of both enzymes, this value matches closely the number and rates of class III and IV hydrogens trapped under similar conditions in phosphorylase *b*. From these results we conclude that the exchange profile of class III and IV reflects rather specifically certain structural requirements of the pyridoxal-P binding site, whereas the faster exchanging classes I and II reflect subunit interactions and are related to dimerization. This interpretation is also consistent with the number of hydrogens (106 hydrogens/monomer) which were trapped in the course of aggregation of the 5′-AMP–apophosphorylase complex. This number and the average rate classes correspond to the class I and II hydrogens trapped during reconstitution with pyridoxal-P. Since an equivalent number of labeled hydrogens is missing if consequently the 5′-AMP–apophosphorylase complex was reconstituted with pyridoxal-P, it appears that the 5′-AMP-dependent dimerization and the pyridoxal-P-dependent dimerization involve identical or overlapping surfaces.

The greater retention of those hydrogens which do not specifically participate in dimerization probably reflect tertiary structure changes. They may be involved in the formation of one (or more) hydrophobic regions, one of which accommodates the cofactor. "Hydrophobic" in this context means restricted access of aqueous solvent. Recent evidence indicates that the pyridoxal-P binding site in phosphorylase is highly nonpolar (Shaltiel and Cortijo, 1970; Johnson *et al.*, 1970; Fischer *et al.*, 1970). The time dependence of the trapping effect shows that not only formerly exposed regions but also parts of the core of the apoenzyme participate in the formation of the newly formed hydrophobic regions. The size might be estimated from the maximal number of hydrogens trapped in classes III and IV. These 230 hydrogens correspond to 120–130 amino acids of the polypeptide chain on the basis of the average amino acid composition of phosphorylase *b* and of the pyridoxal-P binding site (Saari, 1970; Fischer *et al.*, 1970). The number of amino acids involved might be still a minimal estimate, since the percentage of peptide bond hydrogens as compared to other exchangeable sites is probably higher with slow exchanging hydrogens (Englander, 1963, 1967; Rosenberg and Chakravarti, 1968). But thus far only deuterium exchange measured by infrared spectrophotometry can exactly determine the number of exchangeable peptide bonds and allows to express numerically conformational changes in terms of the number of peptide bonds involved (Hvidt and Nielsen, 1966).

In any event, the extent of the structural rearrangement readily explains that certain reactive amino acid side chains, *e.g.*, SH groups, in apophosphorylase become unreactive in holophosphorylase (Avramovic-Zikic *et al.*, 1970). Since the exchange rates of nearly the same number of hydrogens were altered on reconstitution of apophosphorylase with 3′-*O*-methylpyridoxal-P and pyridoxal-P monomethyl ester, one might assume at a first approximation that these analogs cause a similar structural change as pyridoxal-P. However, the slightly faster back-exchange of the 3′-*O*-methylpyridoxal-P derivative indicates that this analog does not stabilize the binding site as effectively as the natural cofactor pyridoxal-P. This is also suggested by the higher reactivity of this analog bound to phosphorylase toward $NaBH_4$ and L-cysteine (Pfeuffer *et al.*, 1972b). The most significant difference between pyridoxal-P monomethyl ester and the enzymatically active analogs is the slight redistribution of fast-exchanging hydrogens, indicating a less stable quarternary structure of this phosphorylase *b* derivative. Pfeuffer *et al.* (1972b) have shown that the quarternary structure of pyridoxal-P monomethyl ester phosphorylase *b* is indeed less stable than holophosphorylase at temperatures >30°. Aside from these differences, the pyridoxal-P monomethyl ester reconstituted from among all the analogs tested a phosphorylase *b* which most closely resembled the native holoenzyme.

The replacement of pyridoxal-P by some analogs shows that the structural requirements of glycogen phosphorylase with respect to its cofactor are neither unique nor absolutely precise. Based on hydrogen exchange one can differentiate between analogs which primarily fail to reconstitute correctly the cofactor binding site and those which are incapable to reassemble and stabilize the native quarternary structure and finally analogs which closely fulfill the steric requirements, but are inactive or less active. One could visualize that interactions which stabilize the protein conformation, such as orientation on the surface of an insoluble carrier, or covalent modification might enhance the activity of a partially active pyridoxal-P analog.

Acknowledgments

We thank Miss E. Pichler and Mr. H. Braun for the preparation of polyethylene glycol [^{14}C]acetate. Mr. H. Feineis built some of the special equipment used in this investigation. The technical assistance of Mrs. I. Eggersdorfer is gratefully acknowledged. To Drs. E. Helmreich and R. H. Haschke we are greatly indebted for a critical review of the manuscript and to Dr. E. Helmreich for suggesting this approach to the study of the pyridoxal-P site in phosphorylase.

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Biosynthesis of Echinulin. Isoprenylation of *cyclo*-L-Alanyl-L-tryptophanyl†

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ABSTRACT: Crude ammonium sulfate fractions of a cell-free extract from *Aspergillus amstelodami* will catalyze the transfer of the 3,3-dimethylallyl moiety from 3,3-dimethylallyl pyrophosphate to *cyclo*-L-alanyl-L-tryptophanyl, forming a possible precursor of echinulin. The reaction product was formed using either a combination of [1-^{3}H]3,3-dimethylallyl pyrophosphate and unlabeled *cyclo*-L-alanyl-L-tryptophanyl or *cyclo*-[^{14}C]-L-alanyl-L-tryptophanyl (labeled on the methylene carbon of the tryptophanyl moiety) and unlabeled 3,3-dimethylallyl pyrophosphate as substrates. Tryptophan, alanyltryptophan, and tryptophanylalanine were not substrates. The chromatographic mobility and chemical reactivity of the enzymic product indicated that it was not echinulin. However, double-radioisotope experiments and ultraviolet and mass spectral analyses indicated that the product was a derivative of *cyclo*-L-alanyl-L-tryptophanyl with a single isoprene substitution on the tryptophanyl moiety. Possible structures of the reaction product are discussed.

A study of the biosynthesis of echinulin (I), a triisoprenylated cyclic dipeptide from *Aspergillus amstelodami*, permits the investigation of a number of unusual biochemical reactions. Although many large cyclic polypeptides have been described, little is known about the formation of cyclic dipeptides. In addition, the alkylation of a tryptophanyl moiety is unusual, particularly the substitution of the 1,1-dimethylallyl grouping at position 2.

Earlier studies (Birch *et al.*, 1961; Birch and Farrar, 1963) have clearly established that the biosynthetic precursors of echinulin are tryptophan, alanine, and mevalonic acid. It has been further established that the L isomer of tryptophan (MacDonald and Slater, 1966) and *cyclo*-L-alanyl-L-tryptophanyl (II)[1] (Slater *et al.*, 1970) are *in vivo* precursors of echinulin. However, no cell-free extracts which are active in catalyzing the incorporation of these precursors into echinulin or any intermediate in its biosynthesis have been described. It is likely that 3,3-dimethylallyl pyrophosphate, a direct prod-

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[1] We have chosen to use the name *cyclo*-L-alanyl-L-tryptophanyl for the diketopiperazine derivative of L-tryptophan and L-alanine instead of *cyclo*-L-alanyl-L-tryptophyl used previously (Slater *et al.*, 1970). The names 3,3-dimethylallyl pyrophosphate and isopentenyl pyrophosphate will be used for 3-methyl 2-butenyl-1-pyrophosphate and 3-methyl 3-butenyl-1-pyrophosphate, respectively.