Quantitative description of the absorption spectra of the coenzyme in glycogen phosphorylases based on log–normal distribution curves

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The absorption spectra of the coenzyme [pyridoxal 5′-phosphate (PLP)] in glycogen phosphorylase *a* (GPha), glycogen phosphorylase *b* (GPhb) and of the latter bound to various effectors and substrates were analysed on the basis of log–normal distribution curves. The results obtained showed that the ionization state of the PLP and GPha environment differs from that of GPhb. This divergence was interpreted in terms of tautomeric equilibria between some forms of the Schiff base of PLP and enzymic Lys-679. The ionic forms are slightly more predominant

in GPha than they are in GPhb, so ionic and/or hydrogen-bonding interactions between the aromatic ring of PLP and GPha must be stronger than with GPhb. This confirms the purely structural role of the aromatic ring of the coenzyme. Binding of GPhb to AMP and Mg^{2+} results in the coenzyme adopting a similar state as in GPha. On the other hand, binding to IMP gives rise to no detectable changes in the tautomeric equilibrium of the coenzyme.

INTRODUCTION

Pyridoxal 5′-phosphate (PLP) is the coenzyme for a wide variety of enzymes, the activity of which thus depends markedly on it. PLP-dependent enzymes include transaminases, decarboxylases and others that catalyse the removal or exchange of β - or γ -substituents between appropriate amino acids, as well as glycosyl-group transfers (e.g. glycogen phosphorylase). In this type of enzyme the coenzyme binds to an ϵ -amino group in an enzymic lysine to form a Schiff base. All these enzymes yield absorption bands in the wavelength range 320–500 nm that are typical of the chromophore in vitamin B₆.

The Schiff bases of PLP occur as different chemical species in solution depending on their pH and environment; these species give different absorption spectra (Metzler et al., 1980; Mitra and Metzler, 1988; Morozov et al., 1988; Vazquez et al., 1991a,b), so the absorption spectra of these bases usually consist of several overlapped bands.

Metzler et al. (1980) showed that the absorption bands of vitamin B₆ derivatives can be accurately described on the basis of log–normal distribution curves. Because the band shapes are highly predictable, spectra of mixed tautomers can be readily resolved into their constituent bands.

The presence of some substrates, inhibitors and effectors or even pH changes can alter the absorption spectrum of many PLP-dependent enzymes in the 320–500 nm region. In dealing with these effects it may be of interest to determine the fractions of the chemical species that yield the different absorption bands. In this respect, deconvoluting enzyme spectra into log–normal distribution curves for PLP-dependent enzymes such as aspartate aminotransferase and glutamate decarboxylase (Kallen et al., 1985; Metzler and Metzler, 1987; Metzler et al., 1988; Miura et al., 1989) was found to be particularly useful for investigating potential reaction intermediates and mechanisms.

Glycogen phosphorylase (1,4- α -D-glucan:orthophosphate α -D-glucosyltransferase, EC 2.4.1.1) catalyses the first step of the intracellular degradation of glycogen, where P_i removes the non-

reducing terminal glucose residue from an α -(1-4)-glycan as α -D-glycopyranosyl phosphate according to the equation:

Glycogen phosphorylase catalyses both the forward and the reverse reaction. The equilibrium constant for the above-mentioned reaction at pH 6.8 is 0.28. Under *in vivo* conditions the phosphate concentration is usually so high that the catalysed reaction is in the direction of glycogen degradation. The structure, function, regulation, role and properties of glycogen phosphorylase have been dealt with in a number of reviews (Fischer et al., 1971; Graves and Wang, 1972; Fletterick and Madsen, 1980; Johnson et al., 1989; Newgard et al., 1989).

Inactive phosphorylase *b* (GPhb) can be activated in two ways, namely (a) by covalent alteration through phosphorylation of serine-14 at its N-terminal tail (glycogen phosphorylase *a*; GPha) in response to hormone and neural signals, and (b) by allosteric binding to the activator AMP.

Glycogen phosphorylase is a special PLP-dependent enzyme inasmuch as its imine double bond can be reduced without the enzyme being rendered inactive (Fischer and Krebs, 1958). This suggests that the aldehyde group of the coenzyme is irrelevant to the catalytic process.

Research into the significance of the different functional groups of the coenzyme in the catalytic process has revealed the phosphate to be the sole group actively involved in the catalytic mechanism; this group is believed to act as a Brønsted acid catalyst (Klein et al., 1982; Klein and Helmreich, 1989) or a Lewis acid catalyst (electrophile) (Withers et al., 1982; Takagi et al., 1982) in the cleavage of the glycoside bond. One alternative interpretation (Johnson et al., 1980) involves the coenzyme phosphate acting as the stabilizing group, which is either a nucleophile that attacks on the face of the sugar ring to displace the phosphate from the substrate or an electrostatic stabilizer for a glycosyl carbonium ion intermediate.

Recently it has been pointed out that likely the unique

Abbreviations used: PLP, pyridoxal 5′-phosphate; GPha and GPhb, glycogen phosphorylases *a* and *b* respectively; Glc1P, α -D-glucose 1-phosphate.

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important role of PLP is to promote the different functions of substrate phosphate, which plays a multiple role: first, it acts as a general acid and transfers a proton to the glycosidic bond, being assisted in this by general acid catalysis by the phospho group of PLP; second, in its anionic form it stabilizes the carbonium ion previously formed and, finally, as a nucleophile it attacked the glycosyl carbonium ion to form Glc1P, and it is again assisted by general basic catalysis by the same group of PLP (Palm et al., 1990; Johnson et al., 1990; Oikonomakos et al., 1992). The very first step in this mechanism is the combination of a PLP dianion with a protonated orthophosphate.

The activation mechanism is associated with a shift in the equilibrium from the low-affinity T-state conformation to the high-affinity R-state (Monod et al., 1965) through ternary and quaternary structural changes promoted by ligand binding. On binding to AMP, phosphorylase *b* induces a change in the state of its coenzyme, which in turn results in a different absorption spectrum with a minimum at 330 nm and a maximum at 360 nm (Donoso et al., 1985). The intensity of such a spectrum is increased by the presence of Mg^{2+} , glucose 1-phosphate and P_i (Buc-Caron et al., 1974; Bresler et al., 1966; Bresler and Firsov, 1968) and has been ascribed to a displacement in the tautomeric equilibrium from the enolimine form to an ion dipole form of the coenzyme (Vázquez et al., 1991b). This change may result from the allosteric activation mechanism or the activation mechanism itself, as it has not been observed in phosphorylase *a*.

Recent X-ray studies on the structural changes undergone by phosphorylase in the activation process, whether covalent or allosteric, revealed that alterations at the catalytic site were quite similar whatever the micromechanism potentially involved (Sprang et al., 1988, 1991; Bardford and Johnson, 1989; Johnson et al., 1990).

In the present paper we analyse the absorption spectra of the coenzyme in inactive phosphorylase, active phosphorylase *a*, and allosterically AMP-activated phosphorylase *b*. The results show that, notwithstanding the qualitative spectral similarities, the coenzyme does not occur in strictly the same ionization state in the two enzymes; also, on binding, AMP displaces the tautomeric equilibrium of its coenzyme to a value closer to that observed in GPha.

EXPERIMENTAL

Materials

GPha and GPhb from rabbit skeletal muscle were purchased in freeze-dried form from Sigma. They were dissolved in glycylglycine buffer and treated with activated charcoal in order to remove AMP (Fischer et al., 1958). The A_{260}/A_{280} ratio measured at pH 6.9 was always less than 0.54. The enzyme concentration was determined by using an $A_{260,1\text{cm}}^{1\%}$ value of 13.2 (Buc et al., 1971). The M_r of the phosphorylase monomer was taken to be 97 500 (Titani et al., 1977).

AMP, IMP, $MgCl_2$ and Glc1P were purchased from Merck. All these reagents except Glc1P, which was treated with activated charcoal in order to retard glycogen formation, were used without further purification.

A solution containing 50 mM glycylglycine, 50 mM KCl and 0.2 mM EDTA at pH 6.9 was used as buffer throughout. The activity of phosphorylase was determined according to the Helmreich and Cori (1964) procedure.

Absorbance measurements were made at $25.00 \pm 0.05^\circ\text{C}$ on a Uvikon 940 spectrophotometer. Differential spectra were obtained by using two-body cuvettes of 1 cm pathlength, except for the (GPha – GPhb) spectrum, which was obtained by subtracting the corrected absorption spectra recorded at the same con-

centration. All other spectra were recorded by using single-body cuvettes and 1 cm pathlength.

Methods

Absorption spectra were deconvoluted by using an IBM PS-II computer and the Peak-Fit (1990) software package to which was added the log-normal function used by Johnson and Metzler (1970).

The log-normal curve in the following form describes the molar absorptivity $\epsilon(\nu)$ at the given wave number ν in terms of the parameters ν_0 , ϵ_0 , W and ρ . ϵ_0 is the wavenumber of maximum molar absorptivity, and W is the width in wavenumbers at half height ($\epsilon_0/2$).

$$\epsilon(\nu) = \epsilon_0 \exp - \left\{ \frac{\ln 2}{(\ln \rho)^2} \left[\ln \left(\frac{(\nu - \nu_0)(\rho^2 - 1)}{W\rho} + 1 \right) \right]^2 \right\};$$

$$\nu > \nu_0 - \frac{W\rho}{(\rho^2 - 1)}$$

$$\epsilon(\nu) = 0; \nu < \nu_0 - [W\rho/(\rho^2 - 1)] \quad (1)$$

The skewness, ρ , is defined as follows:

$$\rho = (\nu_v - \nu_0)/(\nu_0 - \nu_r)$$

where ν_v and ν_r are the two values of wavenumber for which $\epsilon(\nu) = (\epsilon_0/2)$ on the 'violet' and 'red' sides of the curve respectively. It follows that ρ is greater than 1 for curves skewed toward higher wavenumber. As ρ approaches 1.0, the log-normal curve approaches a symmetric Gaussian curve.

The area, a , under $\epsilon(\nu)$ versus ν is given by:

$$a = a_s \epsilon_0 W$$

$$a_s = \sqrt{(2\pi)[\rho/(\rho^2 - 1)]} c \exp(c^2/2)$$

$$c = (\ln \rho)/\sqrt{(2 \ln 2)} \quad (2)$$

a_s varies from 1.06 for $\rho = 1$ to 1.10 for $\rho = 1.5$.

RESULTS AND DISCUSSION

In the absence of effectors, GPhb yields the visible absorption spectrum shown in Figure 1, which shows a tall maximum at 332 nm and a shorter one at 420 nm. This spectrum can be

Figure 1 Absorption spectrum of GPhb at 25°C fitted with log-normal distribution curves ([GPhb] = 13.4 g/l)

a, b and c are single log-normal bands III, II and I respectively, according to Table 1. d is the total spectrum. Lines are theoretical results, and points are the experimental ones.

Table 1 Position and shapes of absorption bands of coenzyme in GPh species

Band	$10^{-3} \times \nu$ (cm^{-1})	λ (nm)	$10^{-3} \times \epsilon_0$ ($\text{M}^{-1} \cdot \text{cm}^{-1}$)	$10^{-3} \times W$ (cm^{-1})	ρ	$10^{-6} \times a$ (mmol^{-1})	a (%)
GPhb							
I	23.78	420	0.46	5.10	1.43	25.7	7.8
II	27.57	363	0.52	3.10	1.04	17.2	5.2
III	30.10	332	5.57	4.67	1.50	286.0	87.0
GPha							
I	23.89	418	0.49	5.10	1.43	27.5	8.3
II	27.57	363	0.71	3.10	1.04	23.2	7.0
III	30.14	332	5.45	4.67	1.50	280.1	84.7
GPhb + AMP + Mg^{2+}							
I	23.83	420	0.47	5.10	1.43	26.3	8.0
II	27.61	362	0.60	3.10	1.04	19.7	6.0
III	30.12	332	5.48	4.67	1.50	281.5	86.0

Scheme 1 Tautomers of PLP **Figure 2** Absorption spectrum of GPha at 25 °C fitted with log-normal distribution curves ([GPha] = 12.0 g/l)

For details, see Figure 1.

ascribed to the Schiff base formed by the coenzyme and enzymic Lys-679. Below 300 nm, the enzyme absorption increases sharply up to a maximum at 278 nm (not shown in Figure 1), due to the protein chromophores, particularly tyrosine and tryptophan residues. A preliminary analysis of the overall spectrum of GPhb revealed that the contribution of the aforementioned chromophores at 310 nm was negligible, so the spectrum was

broken down as shown in Figures 1–4, i.e. between 310 nm ($3.22 \times 10^4 \text{ cm}^{-1}$) and 460 nm ($2.17 \times 10^4 \text{ cm}^{-1}$).

Deconvoluting the spectrum into log-normal curves provided three bands, namely a major band (I) with a maximum at 332 nm, and two minor bands (II) and (III), with maxima at 363 and 420 nm respectively. Band II was a buried band. Table 1 summarizes the spectral features of the three bands and their respective percentage contributions to the overall curve. Fitting of the experimental curve (shown as a dotted line) and its theoretical counterpart (namely the summation of bands I, II and III) was excellent: the residuals never exceeded 0.5%.

According to the results obtained for some Schiff bases of PLP with various amines and amino acids in media of different polarity (Metzler et al., 1980; Mitra and Metzler, 1988; Morozov et al., 1988; Vázquez et al., 1991a,b) and investigations on PLP-dependent enzymes (Kallen et al., 1985; Metzler and Metzler, 1987; Metzler et al., 1988; Miura et al., 1989), the three bands correspond to as many different tautomers of the coenzyme. Such tautomers (Scheme 1) are the zwitterionic form (B_a), the ion dipole form (B_b) and the enolimine (B_c). Both the width and asymmetry of these bands are similar to those of the bands obtained for other PLP-dependent enzymes.

The last column in Table 1 lists the percentage area corresponding to each form, which roughly also denotes the molar fraction of each, as the molar area of the three bands is very similar (Vázquez et al., 1991b). The high incidence of the enol form suggests the occurrence of a highly hydrophobic environment for the coenzyme, consistent with previous findings (Vázquez et al., 1991b; Shaltiel and Cortijo, 1970; Sansom et al., 1984). On the other hand, the fraction of non-enol forms (13%)

is higher than that one would expect on comparing the overall spectra of the holoenzyme and the Schiff base of PLP and *n*-dodecylamine, where the chromophore is embedded in a completely hydrophobic medium (Vázquez et al., 1991b).

The X-ray results (Sansom et al., 1984) appear to indicate the absence of polar interactions between the pyridine nitrogen and any enzyme residue, and that all other interactions with the aromatic ring are of hydrophobic nature, even though certain regions remain to be unambiguously interpreted or may indeed be inherently disordered. Nevertheless, the involvement of these polar forms must be indicative of the occurrence of specific stabilizing interactions.

GPha has a phosphorylated Ser-14 residue and is active in the absence of AMP. The absorption spectrum of its coenzyme is shown in Figure 2. It is qualitatively similar to that yielded by GPhb in the same buffer at the same temperature (25 °C) and pH (6.9). However great this similarity may be, there is no full coincidence. In fact, the deconvoluted spectra show clear dissimilarities that must be related to differences in the coenzyme environment between the active and inactive states. Table 1 shows the spectral features of the three bands into which the spectrum was deconvoluted. The three bands are the same as those obtained for GPhb, namely band I, due to the enolimine form and showing an absorption maximum at 332 nm, band II, yielded by the ion dipole form and having a maximum at 363 nm and band III, corresponding to the zwitterionic form and possessing an absorbance maximum at 418 nm. The differences between the two spectra lie in the disparate contribution of each band to the overall spectrum. The differential spectrum shown in Figure 3 shows changes in the two enzymes more clearly. There is seen to be some bandshift that reflects the displacement in the tautomeric equilibria of Scheme 1 to the ion dipole form, B_b, the contribution of which varies from 5.2% for phosphorylase b to 7% for phosphorylase a, basically at the expense of the enol form, B_c, which accounts for only 84.7% of the latter.

The enol form is more stable in non-polar media involving no strong ionic interactions. Attempts at stabilizing the ion dipole form as the major species of the Schiff bases of PLP by simply changing the medium polarity were unsuccessful, which clearly indicates that this calls for specific ion interactions or hydrogen-bonding. In fact, enzymes where this species is the major one [e.g. aspartate aminotransferase at high pH values (Kallen et al., 1985)], ionic interactions between the protonated nitrogen of the aromatic ring and an aspartate ion are believed to be the source of the stabilizing effect; in addition, phenoxide ion must interact with another two groups to form hydrogen bonds that are bound to provide this form with additional stability.

Visible absorption spectroscopy does not allow the ionization state of the phosphate group in the coenzyme to be determined, since, whether this is present as a mono- or di-anion, the resulting spectrum of the Schiff base is virtually identical (Vázquez et al., 1991a,b). For this reason, none of the structural formulae in Scheme 1 includes the protonation state of the phosphate group. According to ³¹P-n.m.r. results (Feldmann and Helmreich, 1976), it must occur as a monoanion in GPhb.

Addition of AMP to a solution of GPhb under saturation conditions results in a differential spectrum that is similar to, though much weaker than, that shown in Figure 3 as regards the coenzyme region (Donoso et al., 1985). The deconvoluted spectrum of a solution containing 13.4 g/l Phb and 6.5×10^{-4} M AMP was found to be virtually identical with that of GPhb alone within the inherent error margin of the computational method used.

Addition of Mg²⁺ at a final concentration of 1.1×10^{-2} M resulted in an outstanding change in the absorption spectrum of

Figure 3 Difference spectrum between glycogen phosphorylases (GPha-GPhb). The enzyme concentration is 13.4 g/l

Figure 4 Difference spectra between GPhb and GPhb + effectors

a, [GPhb - (GPhb + AMP)]; [AMP] = 6.5×10^{-4} M; b, [GPhb - (GPhb + AMP + P_i)]; [AMP] = 6.5×10^{-4} M, [P_i] = 1.8×10^{-4} M; c, [GPhb - (GPhb + AMP + P_i + Glc1P)]; [AMP] = 6.5×10^{-4} M, [P_i] = 1.8×10^{-4} M, [Glc1P] = 3.0×10^{-4} M. The enzyme concentration was 13.4 g/l.

the coenzyme, consistent with previous findings (Buc-Caron et al., 1974; Bresler et al., 1966; Bresler and Firsov, 1968). Figure 4 shows the differential spectrum obtained in the present study. The overall spectrum yielded by a solution of Phb, AMP and Mg²⁺ was deconvoluted into log-normal curves. The results obtained from them are given in Table 1.

As one would expect from the appearance of the differential spectrum shown in Figure 4, the addition of AMP and Mg²⁺ displaced the tautomeric equilibrium of the coenzyme in the same direction as covalent activation by phosphorylation, i.e. the fraction of form B_b was increased at the expense of form B_c. These results suggest that the allosteric activation of the state reached by PLP is virtually identical with that in which it occurs in GPha.

On the basis of ³¹P-n.m.r. results (Feldmann and Helmreich, 1976; Feldmann and Hull, 1977), AMP has been assigned the role of deprotonating PLP and converting the phosphate group into a dianion. Our results do not allow us to confirm this

hypothesis; however, they do permit us to state that the nitrogen atom of the aromatic ring and the phenol oxygen of PLP must interact with specific residues of the enzyme in the activation process, which would account for the spectroscopic results.

In the other PLP-dependent enzymes (Kallen et al., 1985; Metzler and Metzler, 1987; Metzler et al., 1988; Miura et al., 1989), where the cleavage of the internal imine bond and hence the protonation state of the pyridine nitrogen as an electron sink play a major role in the catalytic process, uptake of the substrates at the catalytic site substantially alters the distribution of the coenzyme tautomers. The little difference between the distribution of these forms in the activated (R) and inactive state (T) in glycogen phosphorylase must be a result of little, or even no, involvement of the aromatic ring of the coenzyme in the catalytic process, so the differences actually found probably suggest that the aromatic ring of PLP plays a merely structural role (Chang et al., 1987). The increased fractions of the ionic forms in the activated state may reflect stronger ionic interactions with other enzyme groups, which may in turn contribute to the phosphate group of the enzyme and the P_i acting as substrate adopting a favourable orientation.

In order to determine the potential effect of substrates on this tautomeric equilibrium we also recorded spectra for 18 mM P_i and 30 mM Glc1P. The corresponding differential spectra are shown in Figure 4. Even though the shape of the three differential spectra shown in the Figure is identical, those yielded by P_i and Glc1P are clearly shifted to positive ΔA regions. In addition, the shift in the Glc1P spectrum increased with time, i.e. as glycogen production developed. Consequently, this spectral distortion can be ascribed to light-scattering caused by the glycogen molecules produced in the reaction, in addition to a potential increase in the concentration of enzyme tetramers brought about by the presence of the substrates (Donoso et al., 1987).

IMP can also slightly activate GPhb with a much smaller binding constant than that of AMP (Mateo et al., 1984). Adding IMP at saturation concentrations to GPhb resulted in no appreciable changes in the visible spectrum of the enzyme. On the basis of X-ray results, Sprang et al. (1991) suggested that the loss of the 6-amino group from the purine ring and its replacement with a oxo group can prevent IMP from forming a fully productive complex with the enzyme, since the 6-amino group in AMP possibly forms hydrogen bonds with the main-chain carbonyl O atoms of residues 315 and 318.

In conclusion, the absorption-spectroscopic results obtained in the present study allow us to state that the allosteric activation of GPhb places the coenzyme PLP in an ionization state and environment that are very similar to those arrived at by covalent activation (GPha) and that the activation process entails reinforcement of ionic or hydrogen-bonding interactions with the pyridine nitrogen and the phenol oxygen of the coenzyme, probably in order to orientate the phosphate group of PLP in the appropriate direction for interacting with the substrates in the catalytic process.

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