THE MODE OF BINDING OF PYRIDOXAL 5'-PHOSPHATE IN GLYCOGEN PHOSPHORYLASE

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Summary. The absorption, fluorescence and chemical properties of the pyridoxal 5'-phosphate (PLP) site in glycogen phosphorylase at pH 7.0 can be simulated by Schiff base derivatives of PLP in non-aqueous solvents. It is proposed that at neutral pH, PLP in phosphorylase is embedded in a hydrophobic microenvironment and bound to an ε-amino group of a lysine residue in the protein through a hydrogen-bonded Schiff base structure.

On the basis of the resemblance between the spectral properties of the PLP† site in glycogen phosphorylase and of conjugates between PLP and amino acids, Kent et al. (1) proposed that, at neutral pH, PLP is bound to the protein through a substituted aldamine structure involving an amino group of the protein and another group, X, which was not identified. It was also suggested (1) that in acidic or alkaline pH, PLP is bound to the protein in a Schiff base structure. These suggestions were supported by the finding that with $NaBH_4$, PLP can be reduced onto the protein at pH 4.0, but not at pH 7.0 (2). We have recently shown that PLP gives rise to a characteristic fluorescence both in the native enzyme (3, 4) and in its $NaBH_4$-reduced form (5). This built-in fluorescent probe provided additional parameters for studying the environment of PLP and its mode of binding in phosphorylase.

MATERIALS AND METHODS

Rabbit muscle glycogen phosphorylase b was prepared, characterized and assayed according to methods described in the literature (6-8). Absorption studies were performed with a Cary Model 15. Fluorescence measurements (corrected excitation and emission spectra) were taken with a Turner 210 "Spectro" (9). Samples used for fluorescence measurements had an optical density below 0.1 at the exciting wavelength. Quantum yields

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† Abbreviations: DMF, N, N'-dimethylformamide; PLP, pyridoxal 5'-phosphate; PMP, pyridoxamine 5'-phosphate.

were determined as described previously (4). In all experiments the temperature was kept at 22° unless otherwise indicated.

RESULTS AND DISCUSSION

The absorption and fluorescence properties of several PLP conjugates were studied in an attempt to find a conjugate that would simulate the PLP site of glycogen phosphorylase and thus clarify the mode of binding of PLP in the enzyme. It was found that the optical and chemical properties of the enzyme can be accounted for if one assumes that PLP is embedded in a hydrophobic milieu and bound to an amino group of the protein in a hydrogen-bonded Schiff base structure.

Absorption Studies — Figure 1A describes the absorption spectra of the enzyme at neutral, acidic and alkaline pH values (1). Above 300 nm the enzyme possesses two absorption bands with maxima at 333 nm and 425 nm (at pH 7.0 $A^{1\%}_{333\,nm} : A^{1\%}_{425\,nm} = 100 : 7.5$). In a neutral aqueous solution there are several conjugates of PLP and amino acids which have an absorption maximum around 330 nm (10, 11). These include conjugates with cysteamine

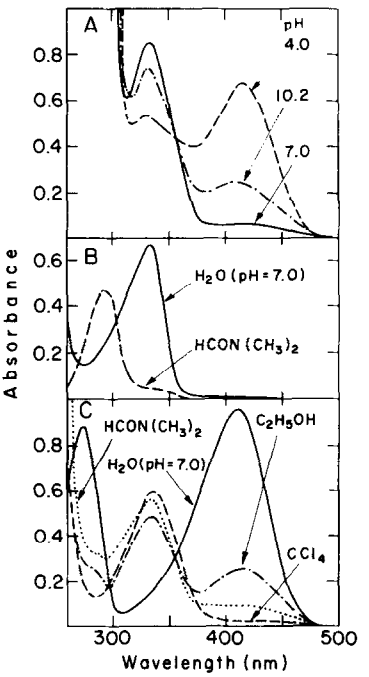

Fig. 1: Absorption spectra of glycogen phosphorylase b and of PLP derivatives. A. Aqueous solutions of the enzyme (15 mg/ml) at various pH values. B. Thiazolidine derivatives of PLP (10^{-4} M) obtained by reaction with an excess of cysteamine (10^{-2} M) in the indicated solvent. C. Schiff base derivatives of PLP (1.1×10^{-4} M) obtained by reaction with an excess of n-hexylamine (10^{-2} M) in the indicated media.

(Fig. 1B), cysteine, histidine and tryptophan. In an organic solvent (e.g. DMF, CCl_4 or ethanol, Fig. 1C), a simple Schiff base conjugate between PLP and n-hexylamine can also simulate the PLP site of phosphorylase: it has a major absorption maximum at 335 nm and a weaker band between 415 and 425 nm. In fact, the relative intensities of these two bands depend on the nature of the solvent. For example, in dioxane-water mixtures the Schiff base has a major peak at 335 nm when the solvent contains 5% water, but it has a major 415 nm peak when the solvent contains 35% water (Fig. 2). Moreover, the transition that occurs upon changing the polarity of the solvent has an isosbestic point at 359 nm (Fig. 2), very similar to the isosbestic point (at 357 nm) that was reported (1) for the titration of the enzyme from pH 7.0 to 4.0.

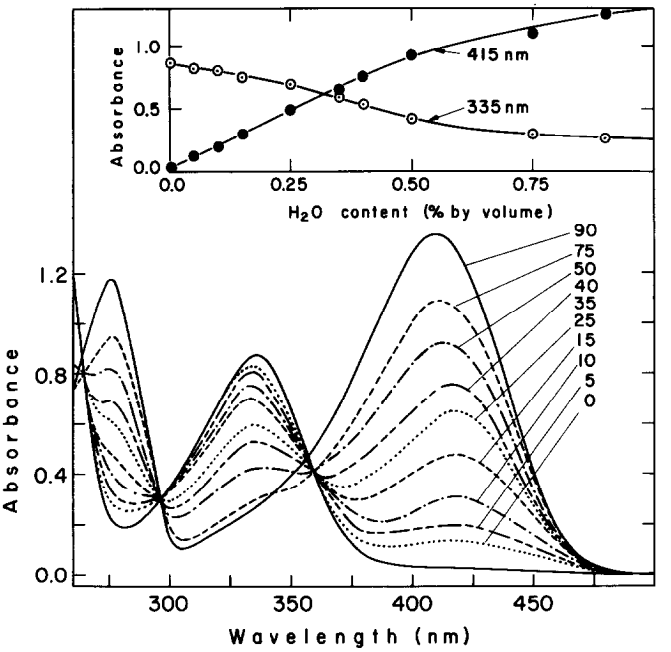

Fig. 2: Absorption spectra of a Schiff base derivative of PLP in dioxane-water mixtures. PLP (1.6×10^{-3} M) was reacted with an excess of n-hexylamine (0.1 M) in absolute dioxane and diluted tenfold into dioxane-water mixtures. The final solutions had the indicated percentage of water (by volume). Inset: Variation of the absorption of the Schiff base at 335 and 415 nm as a function of the water content of the medium.

Fluorescence Studies — **Figure 3 depicts the fluorescence properties of glycogen phosphorylase (A, B and C) as compared with those of a Schiff base derivative of PLP (C, D and E). It is clearly shown that the excitation and emission fluorescence spectra of the enzyme at neutral pH are practically identical with the spectra of the Schiff base in chloroform, but different from the spectra obtained in water, pH 7.0 (Fig. 3). On the other hand, PLP**

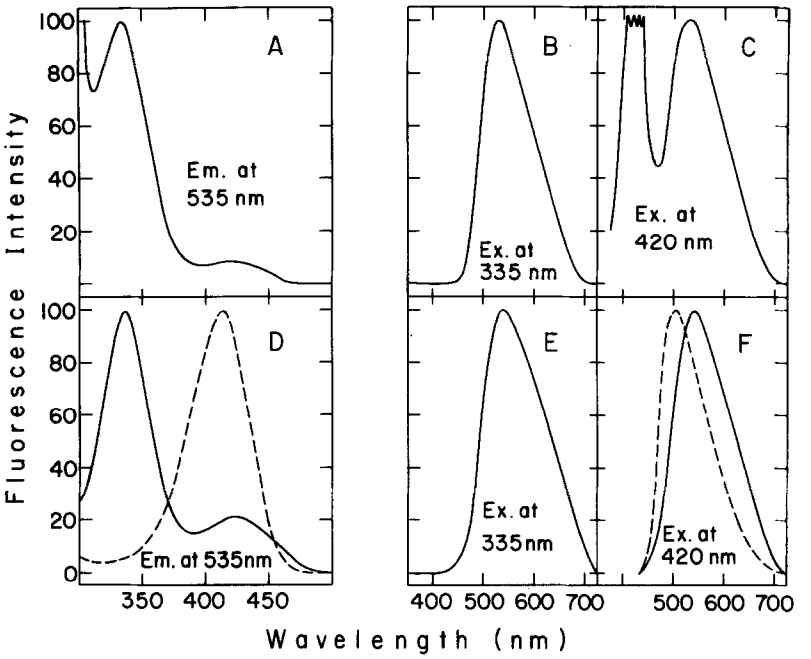

Fig. 3: Fluorescence properties of glycogen phosphorylase b and of a Schiff base derivative of PLP. The enzyme (1.3 mg/ml) was dissolved in a buffer composed of sodium glycerophosphate (0.05 M), 2-mercaptoethanol (0.05 M) and EDTA (10^{-3} M), pH 7.0. The Schiff base was prepared by reaction of PLP (10^{-5} M) with n-hexylamine (10^{-2} M) in either water, pH 7.0 (----) or in chloroform (——). A, excitation spectrum of the enzyme; B and C, emission spectra of the enzyme; D, excitation spectrum of the Schiff base; E and F, emission spectra of the Schiff base.

conjugates which have an absorption maximum at 330 nm in water do not have fluorescence properties similar to the enzyme. Figure 4 illustrates the fluorescence spectrum of a conjugate between PLP and cysteamine. When excited at 330 nm it does not give the 535 nm fluorescence in either an aqueous or a non-aqueous medium. Several other PLP conjugates were screened (with cysteine, homocysteine, histidine, histamine and tryptophan). All of these have absorption maxima at 330 nm (water, pH 7.0) but fluoresce between 390-400 nm.

Reduction with $NaBH_4$ — The model Schiff base derivatives of PLP in an organic solvent (e, g, DMF) can be reduced with $NaBH_4$. However, the rate of reduction in such a medium is much slower than the rate of decomposition of $NaBH_4$ in water. Figure 5 shows the absorption spectrum of a Schiff base derivative of PLP (in DMF) and the spectrum of the product obtained after reduction with $NaBH_4$. It is clearly seen that the reduction is accompanied by a disappearance of the absorption band at 335 nm. This change was used to follow the rate of reduction of the Schiff base derivative of PLP which was found to have

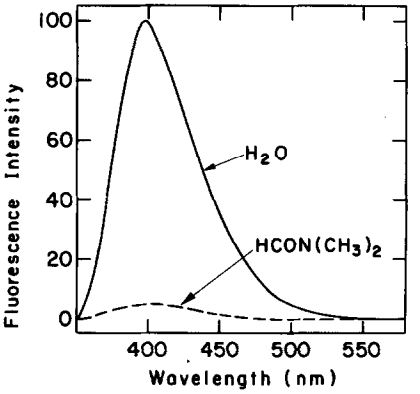

Fig. 4: Fluorescence of a thiazolidine derivative of PLP (excitation at 330 nm). This derivative was prepared by reaction of PLP (10^{-5} M) with an excess of cysteamine (10^{-3} M) either in water, pH 7.0 (——) or in DMF (----).

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a $t_{1/2}$ of 245 sec at 22° (Fig. 5, inset). In an aqueous solution of pH 7.0 (25°) $NaBH_4$ decomposes much faster ($t_{1/2}$ < 12 sec (12)). It is therefore possible that $NaBH_4$ fails to reduce aqueous solutions of phosphorylase at pH 7.0 since the reducing agent decomposes much faster than it can react with the bound PLP in its hydrophobic microenvironment. The low solubility of the BH_4^- ion in this hydrophobic site may also hinder the reduction of PLP onto the protein. It should be noted that under the conditions described in the legend to Fig. 5, the reduction of the same Schiff base in water (pH = 7.0) occurred immediately upon mixing ($t_{1/2}$ < 5 sec).

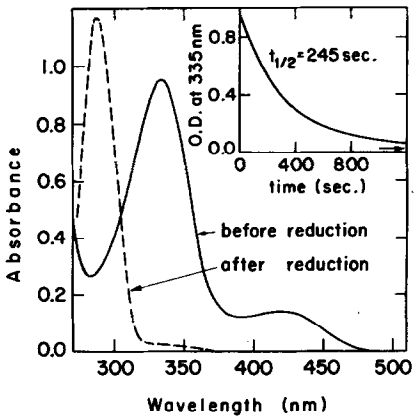
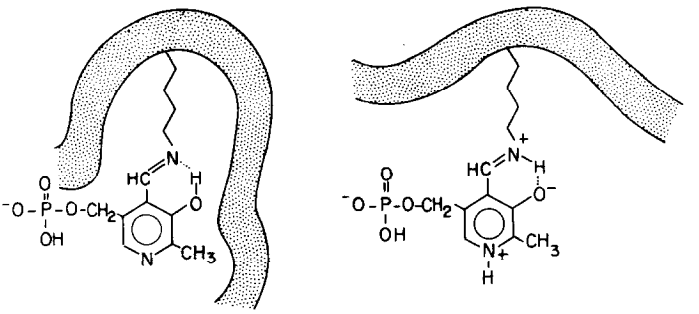


Fig. 5: Reduction of a Schiff base derivative of PLP in DMF. A solution of PLP (3×10^{-4} M) and n-hexylamine (4.5×10^{-2} M) was reduced by addition of 50 moles $NaBH_4$ per mole of PLP (25 µl of a 0.6 M solution of $NaBH_4$ in DMF per ml of the Schiff base solution). The spectrum of the reaction mixture was taken before and after the addition of $NaBH_4$. Inset: Rate of reduction of the Schiff base followed by the drop in absorption at 335 nm.

Proposed Structures for the PLP Site — The evidence presented above clearly demonstrates that a Schiff base derivative of PLP in an organic solvent adequately simulates the PLP site of glycogen phosphorylase in both its absorption and fluorescence properties. Moreover, assigning such a structure to the PLP site of the enzyme can account for the finding reported by Fischer and his associates (2) that PLP cannot be fixed onto the protein by reduction with $NaBH_4$ at neutral pH. On the basis of the above evidence we propose the following structures for the PLP site in glycogen phosphorylase:



Neutral pH

PLP in hydrophobic pocket

Acidic pH

PLP exposed

These structures are analogous to the structures assigned to PLP-Schiff base derivatives in non-aqueous (13, 14) and aqueous (15, 16) media. It should be emphasized that each of the structures assigned to the PLP site is representative of two tautomeric forms (enol-imine and keto-enamine) which characterize PLP-Schiff bases (13). It should also be stressed that the ionization state assigned to the phosphate group is arbitrary, since our data do not necessarily bear upon the microenvironment of the phosphate group.

The structures proposed in the present work can account for several other properties of the PLP site in phosphorylase: 1) The increase in PMP absorption of $NaBH_4$-reduced phosphorylase (330 nm) which occurs upon acidifying a neutral solution of the enzyme to pH 5.5 (17). This transition can be attributed to a conformational change that exposes the PMP residue to a more polar environment (18). Similar increased absorbancies are found in PMP solutions upon increasing the polarity of the solvent (18, 19).

2) The enhancement in the quantum yield of the PMP fluorescence of the reduced enzyme (5). A similar enhancement occurs upon transferring PMP from a non-aqueous solvent into water (18).

3) The increase in the 415 absorption band upon denaturing native phosphorylase with urea at neutral pH (1). This process apparently exposes the PLP residue to a more polar environment.

4) Finally, the proposed hydrophobic environment of the PLP residue could account for the fact that upon removal of PLP from phosphorylase, a sulfhydryl group (presumably at the PLP site) can be selectively labeled with 1-fluoro-2,4-dinitrobenzene (20). This reagent is known to be preferentially adsorbed to lipophilic sites of proteins (21).

The results presented in this paper exemplify the use of the fluorescence, absorption and chemical properties of PLP for elucidating the nature of its microenvironment and its mode of binding to glycogen phosphorylase. A similar approach may be useful in the study of other PLP-enzymes.

REFERENCES

1. Kent, A.B., Krebs, E.G., and Fischer, E.H., J. Biol. Chem., 232, 549 (1958).
2. Fischer, E.H., Kent, A.B., Snyder, E.R., and Krebs, E.G., J. Am. Chem. Soc., 80, 2906 (1958).
3. Shaltiel, S., and Fischer, E.H., Israel J. Chem., 5, 127p (1967).
4. Cortijo, M., Steinberg, I.Z., and Shaltiel, S., J. Biol. Chem., submitted for publication.
5. Cortijo, M., and Shaltiel, S., Biochem. Biophys. Res. Commun., 39, 212 (1970).
6. Krebs, E.G., Love, D.S., Bratvold, G.E., Trayser, K.A., Meyer, W.L., and Fischer, E.H., Biochemistry, 3, 1022 (1964).
7. Shaltiel, S., Hedrick, J.L., and Fischer, E.H., Biochemistry, 5, 2108 (1966).
8. Hedrick, J.L., and Fischer, E.H., Biochemistry, 4, 1337 (1965).
9. Turner, G.K., Science, 146, 183 (1964).
10. Heyl, D., Harris, S.A., and Folkers, K., J. Am. Chem. Soc., 70, 3429 (1948).
11. Buell, M.V., and Hansen, R.E., J. Am. Chem. Soc., 82, 6042 (1960).
12. Gardiner, J.A., and Collat, J.W., J. Am. Chem. Soc., 87, 1692 (1965).
13. Heinert, D., and Martell, A.E., J. Am. Chem. Soc., 85, 183 (1963).
14. Matsushima, M., and Martell, A.E., J. Am. Chem. Soc., 89, 1322 (1967).
15. Metzler, D.E., J. Am. Chem. Soc., 79, 485 (1957).
16. Christensen, H.N., J. Am. Chem. Soc., 80, 99 (1958).
17. Kent, A.B., Ph.D. dissertation, University of Washington, Seattle, Washington (1959).
18. Cortijo, M., and Shaltiel, S., in preparation.
19. Metzler, D.E., and Snell, E.E., J. Am. Chem. Soc., 77, 2431 (1955).
20. Zaidenzaig, Y., and Shaltiel, S., Israel J. Chem., 7, 116p (1969).
21. Green, N.M., Biochim. Biophys. Acta, 74, 542 (1963).