Criteria to Recognize the Structure and Micropolarity of Pyridoxal 5'-Phosphate-Binding Sites in Proteins

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We show that polarography can be a useful tool for the recognition of the mode of binding of pyridoxal 5'-phosphate to proteins. Furthermore we discuss the possibilities of other techniques that can be used to study this subject.

Pyridoxal 5'-phosphate is a necessary cofactor of many enzymic reactions. Almost all these pyridoxal phosphate-dependent enzymes have an absorption band around 415 nm, although most of them have higher peaks at 335 nm. The studies made with simple models have shown that the 415 nm band is due to a Schiff base in a hydrophilic medium [structure (I) of Scheme 1] (Matsushima & Martell, 1967). The 335 nm band can be assigned either to the structure (II) with an sp^2 hybridization on the 4'-carbon atom and the 3-hydroxy group in the phenolic form (Schiff

base in a hydrophobic medium) (Matsushima & Martell, 1967) or to the structure (III) with an sp^3 hybrid and a phenolate form (substituted aldimine in a hydrophilic environment) (Buell & Hansen, 1960). The structure (IV) with both the sp^3 hybrid and the phenolic form has the absorption maximum around 290 nm (Matsushima & Martell, 1967). When a pyridoxal phosphate Schiff base is dissolved in a medium of intermediate polarity, the two bands (415 and 335 nm) are observed and the ratio of these two absorbances can be used as a measure of the medium polarity (Llor & Cortijo, 1977a).

Neither absorbance nor fluorescence measurements can be taken as definitive proof of the mode of pyridoxal phosphate binding. For this reason, we have used polarography as a tool that can give us additional information about the micropolarity of the pyridoxal phosphate site bound to the proteins. We have used as a model the complexes formed between pyridoxal phosphate and bovine serum albumin. These complexes show spectra with absorption maxima at 335 and 415 nm (Dempsey & Christensen, 1962). We provide evidence in the present paper that this technique is sensitive enough to distinguish between hydrophobic and hydrophilic pyridoxal phosphate microenvironments.

Materials and Methods

Crystalline bovine serum albumin from Calbiochem (Lucerne, Switzerland) was defatted as described by Chen (1967). Its concentration was determined spectrophotometrically by using a specific absorption coefficient at 280 nm of $0.66 \text{ litre} \cdot \text{g}^{-1} \cdot \text{cm}^{-1}$ (Dempsey & Christensen, 1962), and a mol.wt. of 67000 (Jonas & Weber, 1971). Pyridoxal phosphate was obtained from Merck (Darmstadt, Germany), L-cysteine was from Sigma (St. Louis, MO, U.S.A.) and NaBH_4 from Carlo Erba (Milan, Italy). Other chemicals were of the purest grade commercially available. Pyridoxal phosphate Schiff bases were formed by

Scheme 1. Possible structures for pyridoxal phosphate bound to proteins, indicating the absorption maxima and the environment ('shaded' area indicates a hydrophobic medium)

addition of a 100-fold excess of *n*-hexylamine to pyridoxal phosphate solutions.

The absorbance, fluorescence and polarographic measurements were made with Coleman 55, Aminco and Radiometer PO4 instruments respectively.

Results and Discussion

Some of the most interesting polarographic results, in water and in non-aqueous solvents and with simple models, are summarized in Table 1. According to the last column of this Table, similar behaviour can be expected when NaBH_4 is added to pyridoxal phosphate Schiff bases and the aqueous substituted-alimine solutions. In fact, both the pyridoxal phosphate Schiff bases and the cysteine-pyridoxal phosphate complexes are reduced with aqueous NaBH_4 (J. S. Jimenez, J. Llor & M. Cortijo, unpublished work), indicating that the conclusions obtained from the lack of reduction of pyridoxal phosphate bound to some enzymes with NaBH_4 are dubious. Perhaps the accessibility of NaBH_4 to the site and the equilibrium constants play an important role at this point.

As was stated in the introduction, the absorption spectra of pyridoxal phosphate bound to proteins can indicate the mode of binding of this vitamin only if the microenvironment of the pyridoxal phosphate site is known. The results given in the last column of Table 1 indicate that it is possible to evaluate the polarity of the pyridoxal phosphate site in proteins from polarographic measurements. When pyridoxal phosphate bound to proteins in a hydrophilic microenvironment is studied by polarography, a wave around -0.7V must be obtained (see Table 1). The half-wave potentials ($E_{\frac{1}{2}}$) become more negative with increasing pH, reaching -1.0V at pH values higher than 9.5 (Llor & Cortijo, 1977b). Therefore the absence of any reduction wave at more positive potentials than -1.0V must imply that the pyridoxal phosphate microenvironment should be either

hydrophobic or very basic. We do not know of any studies indicating the presence of very basic pockets formed by proteins dissolved at a not very basic pH.

It could be argued that the equilibrium of the structures present in the bulk solution could be shifted towards the most easily reducible one on the mercury electrode. This is a well-known fact in polarography whenever the rate constants of inter-conversion between the two forms are faster than the lifetime of the electrode (usually 2 or 3s). However, with Schiff bases (or substituted aldimines), formation takes from several minutes (Llor & Cortijo, 1977b; Buell & Hansen, 1960) to several hours (Dempsey & Christensen, 1962). This objection should therefore be disregarded.

We have therefore studied the polarographic behaviour of the two complexes of bovine serum albumin and pyridoxal phosphate (sites I and II) (Dempsey & Christensen, 1962). The results at neutral pH are given in Fig. 1(c), with a wave at -0.80V for the 1:2 complex. This wave has a diffusion character and it can be easily ascribed to the reduction of pyridoxal phosphate bound to the site II. Its half-wave potential changes with pH in a way similar to that shown by a single Schiff base in an aqueous medium. The intensities decrease with increasing pH, indicating that the diffusion constant of pyridoxal phosphate decreases, i.e. that this molecule is bound more tightly at neutral than at acidic pH. It can be argued that this wave was due to the reduction of free pyridoxal phosphate dissociated from the protein. Several arguments can be made against this possibility. (1) The equilibrium constant is 10^5 mol/litre in this case (Dempsey & Christensen, 1962). (2) The half-wave potentials of pyridoxal phosphate and its Schiff base are different (Llor & Cortijo, 1977b). However, this cannot be viewed as evidence, because these potentials change with the pH, and the micro pH of the pyridoxal phosphate site is unknown. (3) With simple models the rate constant of the Schiff-base dissociation is slower than the lifetime of the electrode (Llor & Cortijo, 1977b).

Table 1. Absorption and polarographic properties of pyridoxal phosphate Schiff bases and substituted aldimines in polar and non-polar media

Abbreviations used: DMSO, dimethyl sulphoxide; DMF, *NN*-dimethylformamide; $E_{\frac{1}{2}}$, half-wave potential.

Structure*	Pyridoxal phosphate in the presence of:	Solvent	Absorption band (nm)	$E_{\frac{1}{2}}$ (V)†
(I)	Hexylamine	Water, pH 7	415	-0.63
(II)	Hexylamine	DMSO or DMF	335	-1.51
(III)†	Cysteine	Water, pH 7	335	-0.75
(IV)†	Cysteine	DMSO or DMF	290	-1.45

* See Scheme 1.

† The cysteine-pyridoxal phosphate and propylenediamine-pyridoxal phosphate complexes give practically the same results.

‡ Measured against a saturated-calomel electrode. Values are taken from Llor *et al.* (1977, 1978).

Fig. 1. Polarograms of bovine serum albumin (a) and its adducts with pyridoxal phosphate (1:1, b; 1:2, c). The adducts were formed in a 0.2M-lithium phosphate buffer, pH7.0 (concn. 3.3mM). After 24h the three samples were diluted 7-fold in the same buffer and the polarograms were recorded against the saturated-calomel electrode.

(4) Even if this rate were faster with proteins, the reduction wave should be kinetic, and we have observed that it shows a diffusion character. We take this case as an example where the wave is observed at potentials more positive than -1.0V , owing to the hydrophilic character of the pyridoxal phosphate-binding site. This site can then be represented by the structure (I) given in Scheme 1. A similar example has been reported by Scheller & Will (1973) with glycogen phosphorylase at slightly acidic pH values.

The polarographic reduction of the 1:1 complex (Fig. 1b) shows an ill-shaped wave around -1.2V . The intensity of this wave is not proportional to the complex concentration, and it changes irregularly with the height of the mercury head. These facts appear to indicate that this wave could be caused by the charge of the double layer around the mercury drop, although a pre-wave caused by the discharge of protons of the adsorbed protein cannot be ex-

cluded. In any case the most important fact is the absence of any reduction wave at potentials more positive than -1.0V . This absence indicates the impossibility of a hydrophilic environment for the site I. As this complex has an absorption maximum at 335nm we must conclude that the structure (II) (Scheme 1) is present in the pyridoxal phosphate bound to the protein in the site I. The discharge of protons starts at -1.4V (even with albumin alone), precluding the detection of the possible wave due to the reduction of the pyridoxal phosphate in site I (Fig. 1a).

Shimomura & Fukui (1977) published circular-dichroism data supporting the same structure for site I. Absorbance and fluorescence measurements also appear to support this structure (J. S. Jimenez, J. Llor & M. Cortijo, unpublished work).

The electrode was actually in an apolar solution in the studies with model compounds. We believe that this is an adequate model for the reduction of the pyridoxal phosphate in a hydrophobic microenvironment of the protein in aqueous solution, since we always had very low electric resistances in the model-compound solutions (Llor *et al.*, 1977a). However, we are looking for a better model that would simplify this problem.

The differences between the structures of the pyridoxal phosphate adducts in hydrophilic and hydrophobic media (given in Table 1) are mainly due to the differences in the protonation of the phenolic group, which is protonated in an apolar solvent and unprotonated in aqueous solution (Scheme 1).

The forms in which the phenolic group are protonated have far more negative half-wave potentials (Table 1) than has the anodic wave of the NaBH_4 (-0.64V), which is responsible for the reductive power of this compound (Gardiner & Collat, 1965). Therefore these forms should not be reduced with this chemical. The suggestion by Schellenberg (1963) that the tertiary iminium salt or the protonated Schiff bases are actually reduced by BH_4^- is then proved. These phenolate forms are easily reduced with NaBH_4 , although there is evidence in the literature that structure (III) is not hydrogenated over a platinum catalyst (Heyl *et al.*, 1948).

The extrapolation of this fact to the reduction (or non-reduction) of pyridoxal phosphate-dependent enzymes can lead to erroneous conclusions, because neither the concentrations at equilibrium nor their reaction rates as given in Scheme 1 are known for the enzyme complex. Therefore we propose that reduction of pyridoxal phosphate bound to a protein by NaBH_4 should not be taken as a criterion of a definitive structure of the site.

We indicate here some other modes of approach to solve this problem. Fluorescence proved to be a very useful technique with phosphorylase (Shaltiel &

Cortijo, 1970), but each case should be considered independently, since fluorescence is very sensitive to changes in the neighbouring group in the site, as was shown by Arrio-Dupont (1971) with aspartate transaminase. The ^{13}C n.m.r. spectroscopy with pyridoxal phosphate labelled in the 4'-position appears to be a very sensitive indicator of the hybridization of this carbon atom. With D-serine dehydratase the chemical shifts corresponded to a Schiff-base structure at both pH 7.8 and 9.4 (O'Leary & Payne, 1976), whereas their u.v. spectra are significantly different. Unfortunately these resonances are not visible with other high-molecular-weight enzymes such as glutamate decarboxylase (O'Leary & Payne, 1976). The polarographic approach used here may be useful in the study of the microenvironment and mode of binding of pyridoxal phosphate residues in other proteins. Thus polarography was a satisfactory technique corroborating the structure of the pyridoxal phosphate-binding site in phosphorylase (Cortijo *et al.*, 1976).

At this point we believe that a Schiff base is the most probable, and possibly the only, structure in pyridoxal phosphate-dependent enzymes. We are, however, aware of the risk involved in using an inductive method based on a small number of cases. Nevertheless, we think it is very improbable that the distances and angles in the tertiary structure of the protein could be suitably rearranged to form a substituted aldimine. In fact the stability and the kinetics of formation of these structures are strongly dependent on the chemical nature of the ring substituents. This has been demonstrated with several analogues of cysteine (Buell & Hansen, 1960).

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