A BUILT-IN FLUORESCENT PROBE IN $NaBH_4$-REDUCED GLYCOGEN PHOSPHORYLASE

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Summary. The pyridoxamine 5'-phosphate residue in $NaBH_4$-reduced glycogen phosphorylase b can be regarded as a built-in fluorescent probe in the enzyme. The fluorescence of this probe was used to demonstrate that around pH 6.1 - 6.2 there is a structural change in the enzyme that occurs concomitantly with the loss of its catalytic activity. This finding could indicate that pyridoxal 5'-phosphate is either at the active site of phosphorylase, or at a site the structure of which is closely associated with that of the active site.

PLP† confers upon glycogen phosphorylase a green fluorescence (Shaltiel and Fischer, 1967) which can be used for studying the structure of the PLP site and for the detection of structural changes in the enzyme. Since PLP in phosphorylase may be bound in two different interconvertible forms (Kent et al, 1958) and since each of these has a different type of fluorescence, the interpretation of PLP fluorescence data in native phosphorylase may sometimes be complicated. Yet when phosphorylase is reduced with $NaBH_4$ (Fischer et al,1958a) PLP is converted to a PMP residue which

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† Abbreviations: PLP, pyridoxal 5'-phosphate; PMP, pyridoxamine 5'-phosphate

is attached to the protein through a definite, non-hydrolyzable type of binding. The changes in the fluorescence of the PMP residue will now reflect changes in its environment and there will not be an interference due to changes in the mode of binding of the cofactor. Moreover, reduction with $NaBH_4$ is a particularly useful modification of phosphorylase since the resulting modified enzyme is catalytically active. Therefore, changes in its fluorescence can be directly correlated with changes in its activity.

This communication reports the characteristics of the PMP fluorescence of $NaBH_4$-reduced phosphorylase b and illustrates its use for a correlation between catalytic activity and structural changes in the enzyme.

MATERIALS AND METHODS

Crystalline glycogen phosphorylase b was prepared by the method of Fischer et al (1958b), freed from AMP by passage through a charcoal-cellulose column (Fischer and Krebs, 1958) and assayed by the method of Hedrick and Fischer (1965). Enzyme concentration was determined spectrophotometrically using an absorbancy index $A^{1\%}_{278}$ 11.9 (Appleman et al, 1963). $NaBH_4$-reduced phosphorylase b was prepared as described by Strausbauch et al (1967).

Absorption spectra were taken with a Cary Model 15. Fluorescence measurements (excitation and "true" emission spectra) were performed using a Turner 210 "Spectro" with a thermostated cell holder at 30^0. Enzyme solutions used for spectral and fluorescence studies were always in sodium β-glycerophosphate (5×10^{-2}M), 2-mercaptoethanol (5×10^{-2}M), EDTA (10^{-3}M), adjusted to the indicated pH value with HCl.

RESULTS AND DISCUSSION

The PMP residue in $NaBH_4$-reduced glycogen phosphorylase has a characteristic fluorescence, with an excitation maximum at 330 nm and an emission maximum at 392 nm (at pH = 7.0). At this pH the PMP fluorescence of the enzyme has a very low quantum yield (ca 0.015) as compared with that of free PMP (0.14, Chen, 1965). However, upon acidification of the enzyme solution there is a remarkable enhancement in its fluorescence with practically no shift in the emission maximum (figure 1).

Kent (1959) had observed that acidification of $NaBH_4$-reduced phosphorylase solutions causes an increase in the absorption of the enzyme around 330 nm. Since such an increase in absorption could give rise to the enhanced fluorescence, a detailed spectrophotometric titration of reduced phosphorylase was conducted and

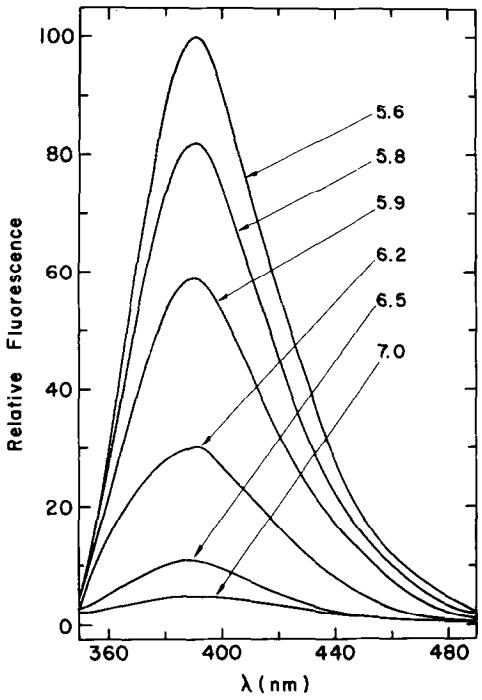

Fig. 1: Spectrofluorimetric titration of $NaBH_4$-reduced glycogen phosphorylase b. Protein concentration: 4 mg/ml, and pH as indicated in the figure. Excitation at 330 nm.

these absorption spectra were used to calculate the quantum yield of the PMP fluorescence as a function of pH (figure 2). It was thus demonstrated that the enhancement in the fluorescence is not due merely to an increased absorption.

As seen in figure 2, at pH 5.8 the quantum yield of the PMP fluorescence rises to a value of 0.11, close to the one reported for free PMP. In the region between pH 8 and 5.5 there is no change in the fluorescence intensity of free PMP. The dramatic change observed in the enzyme is therefore associated with the particular environment of the PMP residue in the protein.

Concomitant with the enhancement of the quantum yield, there is a decrease in the catalytic activity of the reduced enzyme (apparent pK of 6.1-6.2 in both cases). If this is not due to a coincidence, such a correlation would indicate that PLP is either at the catalytic site of the enzyme, or at another site the structure of which is intimately associated with that of the active site.

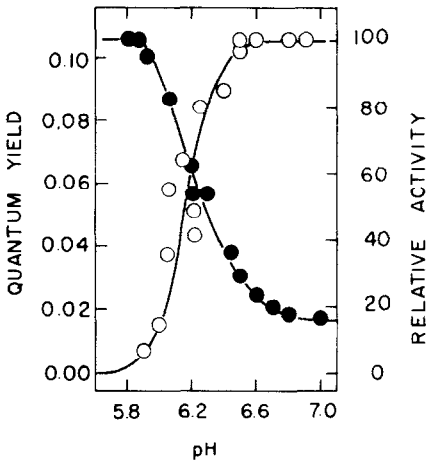

Fig. 2: pH-Dependence of the catalytic activity (—○—) and the quantum yield of the PMP fluorescence (—●—) in $NaBH_4$-reduced glycogen phosphorylase b Excitation of the fluorescence at 330 nm.

It should be noted that both the activity and the fluorescence of the enzyme have rather steep pH dependence curves (figure 2). These abrupt transitions (within 0.8 pH units) are suggestive of a cooperative ionization involving more than one functional group. This process could involve, for example, the protonation of the nitrogen atom in the pyridine ring of PMP. The involvement of this nitrogen atom in catalysis was recently suggested by Bresler and Firsov (1968).

The effect of pH on the activity and the fluorescence of the enzyme could also be associated with structural changes resulting from the ionization of the phosphate group in PMP. Such a claim could be supported by the fact that the transition occurs around pH 6.1, close to the pK of the phosphate group in PMP (Jencks and Regenstein, 1968). This phosphate group seems to be indispensable to the catalytic activity of the enzyme since its replacement by other groups has so far resulted in PLP analogs incapable of restoring activity to apophosphorylase (Shaltiel et al, 1969). The possible participation of this phosphate group in catalysis has already been raised by Kastenschmidt et al, (1968).

It is not possible at this stage to determine which functional groups take part in this process and how the protein moiety is involved. In any case the observations reported in this communication provide a sensitive physicochemical parameter for the study of structure-function relationships in glycogen phosphorylase.

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