A POLAROGRAPHIC STUDY ON PYRIDOXAL 5'-PHOSPHATE BINDING IN GLYCOGEN PHOSPHORYLASE *b*

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1. Introduction

The function of the cofactor, pyridoxal 5'-phosphate (PLP), and the structure of its binding site in glycogen phosphorylase *b* (α-1.4 glucan:orthophosphate glucosyltransferase, EC 2.4.1.1) are subjects of intensive investigations. In the native enzyme, PLP is found to be buried in the hydrophobic region, from which it is transferred into the aqueous phase by acidification [1]. The binding between cofactor and apophosphorylase is achieved by the C_4-aldehyde group of PLP and the ε-amino group of a lysyl residue [2]. While the Schiff's base structure seems to be reasonably documented for the acidic pH region [1, 3, 4], substituted aldamine has been postulated for the neutral region as an alternative [4, 5].

The present paper reports on first results of the electrochemical reduction of glycogen phosphorylase *b*. It is shown that phosphorylase *b*, in the acidic region (below pH 5.4) in contrast to the neutral region gives a characteristic d.c. polarographic step as a result of the reduction of the linkage between PLP and the apophosphorylase.

2. Materials and methods

Phosphorylase *b* was extracted from rabbit skeletal muscle according to the method of Fischer and Krebs [6] with the modification that L-cysteine HCl was replaced by β-mercaptoethanol. The enzyme was recrystallized 4–5 times from the buffer: 0.05 M Na-β-glycerophosphate–0.001 M mercaptoethanol–0.001 M AMP–0.01 M $Mg(CH_3COO)_2$, pH 6.8. In order to adjust the desired pH value, phosphorylase *b* was either titrated, after dialysis against 0.05 M Na-β-glycerophosphate–0.0001 M EDTA buffer, pH 6.8, directly in the measuring cell with 0.1 M CH_3COOH and 0.1 M HCl, respectively, or was filtrated prior to polarographic measurements through a Sephadex G-25 column (3 × 40 cm) equilibrated with 0.05 M Na-β-glycerophosphate–0.0001 M EDTA buffer of the desired pH value. Protein was either determined by the method of Lowry et al. [7] with serum albumin as the standard reference, or by direct spectrophotometric measurement with the absorbancy index for phosphorylase *b* of $A_{280}^{1\%} = 13.2$ [8]. Enzyme molarity was calculated on the basis of a molecular weight of 200,000 for the dimeric phosphorylase [9].

Polarographic measurements were made with the polarograph GWP 563 of the Akademiewerkstätten Berlin-Adlershof with a thermostated measuring cell (20°). The drop time of the mercury electrode was 3–4 sec. A chargeable saturated calomel electrode was employed as the counterelectrode. Before each measurement, oxygen was removed from the solution by a slow stream of nitrogen. 0.1 M KCl was used as the conducting electrolyte.

AMP and Na-β-glycerophosphate were purchased from Boehringer GmbG, Mannheim; mercaptoethanol, extra-pure, was supplied by Serva Entwicklungslabor Heidelberg, and EDTA, p.a., by VEB Berlin-Chemie.

3. Results and discussion

The polarographic reduction of the aldehyde group of PLP was found to provide a well-defined d.c. polarographic step (fig. 1) with nearly constant height between pH 2 and pH 8 [10]. The half-wave potential ($E_{1/2}$) is a function of the pH value, because protons participate in the reduction reaction. For the pH range 2–8 we found the equation:

$$E_{1/2} = -(0.320 + 0.060\ pH)V \qquad (1)$$

In aqueous solutions, if a large excess of amine compounds is present, PLP is almost completely converted into the corresponding Schiff's base [11]. In general, these azomethines are reduced at more positive potentials than the free aldehydes [12].

In order to simulate the PLP linkage in the phosphorylase we investigated the polarographic behaviour of PLP azomethines. These Schiff's bases formed with *n*-butyl amine, γ-aminobutyric acid, and valine, show throughout the pH region investigated half-wave potentials which were maximally 60 mV more positive than for free PLP. These small differences exclude a polarographic differentiation between free PLP and its Schiff's bases (fig. 1).

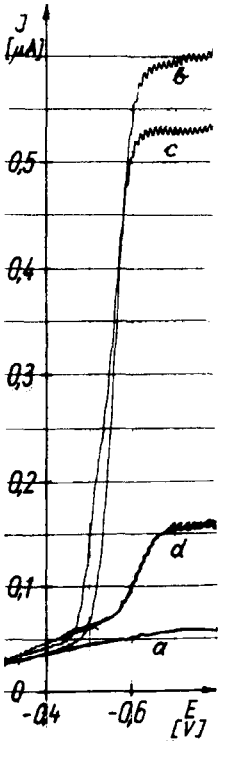

Fig. 1. Polarographic current–voltage curves. a) Background solution: 5×10^{-2} M Na-β-glycerophosphate; 10^{-4} M EDTA, pH 4.9. b) 10^{-4} M PLP, pH 4.9. c) 10^{-4} M PLP + 2×10^{-1} M γ-amino-butyric acid, pH 4.9. d) 7.3×10^{-5} M glycogen phosphorylase *b*, pH 4.9.

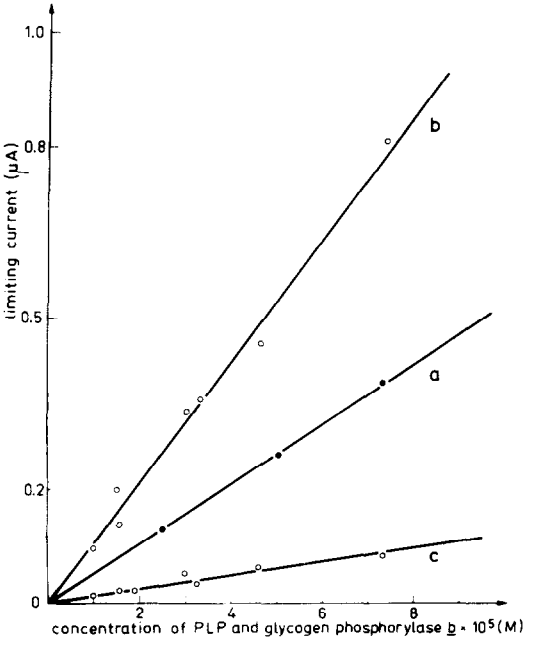

Fig. 2. Concentration dependence of cathodic limiting currents. a) PLP. b) Glycogen phosphorylase *b*, pH 2.0. c) Glycogen phosphorylase *b*, pH 4.9.

In the neutral pH region, there was no reduction current for native dimeric glycogen phosphorylase *b* containing one molecule PLP per subunit, even at high enzyme concentration. But a cathodic step was observed below pH 5.4, the half-wave potential of which nearly coincided with that of free PLP or its amine adducts (fig. 1). The magnitude of limiting current, contrary to free PLP, was pH-dependent and reached its maximum value on direct acid titration of the enzyme solution at pH 2. According to Kent et al. [3], PLP binding is hydrolyzed below pH 4. Therefore, on titrating in this region a mixture of native phosphorylase *b*, apophosphorylase and free PLP is present, until all PLP is liberated from the phosphorylase at pH 2. In fact, the limiting current at pH 2

corresponds to two moles of PLP per mole phosphorylase as could be anticipated for the native enzyme (fig. 2).

Using Sephadex filtration (see Materials and methods) in the region pH 5.4–4.8 we were able to exclude a dissociation into PLP and apophosphorylase, so that the observed cathodic wave reflected the reduction of the PLP binding. By contrast, Sephadex filtration at pH 4.5 showed the dissociation of enzyme into PLP and protein. The apophosphorylase did not show a cathodic wave.

In order to characterize the polarographic step of phosphorylase, the concentration dependence of the limiting currents of free PLP and the holoenzyme, adjusted to pH 4.9 by gel filtration, was studied (fig. 2). According to Ilkovic's equation [13]

$$I_{lim} = k \cdot c \cdot n \cdot D^{1/2} \qquad (2)$$

the limiting current I_{lim} is linearly dependent upon concentration c in both cases (fig. 2). (Equation 2: k = proportionality constant, n = number of electrons per molecule picked up at the electrode, D = diffusion constant). Obviously, the slope m of the two straight lines obeys the relationship:

$$\frac{m_{phosphorylase}}{m_{PLP}} = \frac{n_{phosphorylase} \cdot D^{1/2}_{phosphorylase}}{n_{PLP} \cdot D^{1/2}_{PLP}} \qquad (3)$$

From fig. 2 we obtained for $m_{phosphorylase}/m_{PLP} = 0.23$. The diffusion constant of PLP was determined to be $D_{20} = 4.9 \times 10^{-6}$ cm^2/sec [14], while for the dimeric phosphorylase a value of $D_{20} = 4.14 \times 10^{-7}$ cm^2/sec has been used [2]. Consequently, the ratio $n_{phosphorylase}/n_{PLP}$ is ~ 0.8. This means that on the average about as much electrons are picked up per one molecule dimeric phosphorylase* as per one molecule of free PLP.

Obviously, on titrating phosphorylase *b* in the region of pH 5, the enzyme undergoes a conformational change: while there was no reduction current observed up to pH 5.4, there is a cathodic wave from this pH downward, the height of which increases down to pH 4.9. This is in accordance with the results obtained by reduction of the enzyme with $NaBH_4$. Phosphorylase *b* can be easily reduced with $NaBH_4$ at pH 4, but hardly so at pH 7 [16, 17]. Recently, Cortijo et al. [4] have reported the pH dependence of the 535 nm fluorescence of PLP in phosphorylase *b*, stating that the observed reversible quenching of fluorescence at pH 5.4 (and pH 9.6) arises from a structural change of the PLP binding site. The fact, that on the average only one of the two linkages between cofactor and protein is reduced cathodically at pH 4.9 may be caused by different reasons:

i) Provided that both of the PLP linkages in one phosphorylase molecule are reduced, the postulated conformational transition may not be complete at pH 4.9 so that under these conditions a part of the enzyme molecules may be present in the conformation characteristic for the neutral region and thus is not reducible. Judging from fluorescence extinction at pH 4.9 [4], however, this part can only make about 10%. The dissociation into PLP and apophosphorylase does not permit that the limiting current of the holoenzyme may be determined and thus to observe the conformational change at lower pH values.

ii) It is also possible that at pH 4.9 both of the PLP linkages are potentially reducible but only one of them is available for the electrode process while the other one is placed in the back of the molecule.

iii) Finally under these conditions a stable state of the enzyme may exist in which only one PLP linkage is exposed as a Schiff's base [18].

* According to Livanova et al. [15], up to pH 4.0 no dissociation of the enzyme into its subunits was observed.

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