NEGATIVE COOPERATIVITY IN PYRIDOXAL 5'-PHOSPHATE BINDING AND ENZYME ACTIVITY OF 6-PHOSPHOGLUCOSE ISOMERASE FROM *BACILLUS CALDOTENAX*

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1. Introduction

Schnackerz and Noltmann [1] demonstrated using pyridoxal 5'-phosphate (PLP) as a specific modifier of lysine residues that 6-phosphoglucose isomerase (EC 5.3.1.9) from rabbit muscle contains one essential lysine residue/subunit and that the two PLP-binding sites in the enzyme are equivalent and independent. The isomerase from *Bacillus caldotenax* consists of 4 identical subunits [2]. It was also found that the thermophilic isomerase was specifically inhibited by PLP and that the PLP-binding was cooperative. Negative cooperativity in PLP-binding and also in enzyme activity of the *B.caldotenax* isomerase is reported here.

2. Materials and methods

Phosphoglucose isomerase was purified from *B. caldotenax* YT-G according to [2].

PLP, when added to a protein, is reversibly bound to the amino groups of the lysine residues in the protein, forming a Schiff base, and on reduction of the Schiff base with borohydride, PLP is irreversibly bound to the amino groups [3]. The thermophilic isomerase (1.4 mg/ml) in 25 mM acetate buffer (pH 6.0) was incubated with 0.02–4.9 mM PLP (3–720 mol PLP/mol enzyme) at 0°C for 15 min and the Schiff base thus formed was reduced with 20 mM sodium borohydride for 15 min at 0°C, according to [1]. On addition of borohydride, the absorption peak around 410–420 nm of the Schiff base disappeared instantaneously. Immediately or 15 min after the addition of borohydride, the sample was diluted or dialyzed against 25 mM acetate buffer (pH 6.0), respectively, and assayed. The enzyme activities of these two samples were almost the same. The results shown above indicate an instantaneous completion of the borohydride reduction of the Schiff base. As an example, the binding of PLP to the isomerase is shown in fig.1. to proceed to equilibration for the 15 min incubation. The amount of PLP bound/enzyme molecule was determined spectrophotometrically at 325 nm using a molar extinction coefficient of 10 000 M^{-1} cm^{-1} for N^6-phosphopyridoxyllysine [1].

Enzyme was assayed in 50 mM Tris–HCl buffer (pH 7.0) at 65°C by determining colorimetrically the phosphofructose (F6P) formed in a forward reaction (colorimetric assay) or at 30°C by determining F6P formation [4] or the amount of phosphoglucose (G6P) formed in a backward reaction [5], employing a coupled spectrophotometric assay (coupled assay). When enzymes treated with PLP were assayed, the enzymes modified as above were dialyzed against 25 mM acetate buffer (pH 6.0) at 4°C for 12 h. Fluorescence measurements were performed in 50 mM Tris–HCl buffer (pH 7.0) with a Shimadzu RF-502 recording spectrofluorimeter at room temperature.

The intracellular concentrations of G6P and F6P in the organism were determined with the cells in a middle logarithmic growth phase, according to [6].

Protein concentration was determined as in [7], using crystalline bovine serum albumin as a standard.

3. Results and discussion

The relationship between the remaining activity and the number of PLP molecules bound/mol enzyme for the rabbit muscle isomerse was linear [1]. The extrapolation of the straightline intersected the axis for the no. mol PLP bound/mol enzyme at 2. Since the isomerase consists of 2 identical subunits, it was concluded that the enzyme contains 2 PLP-binding

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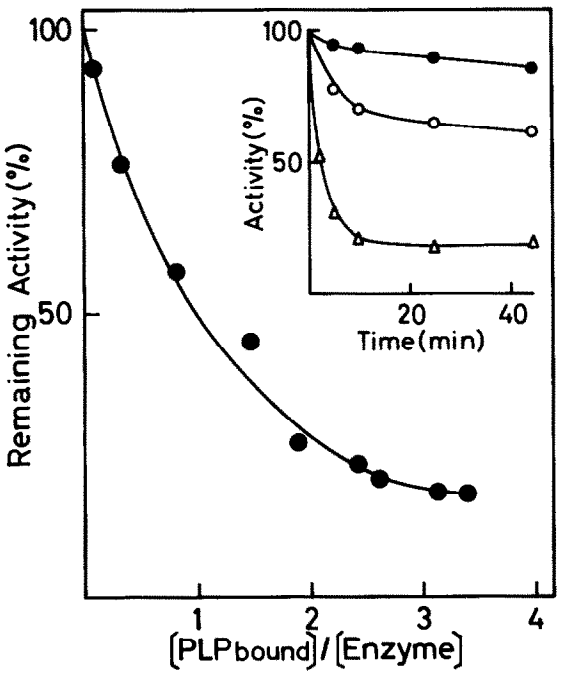

Fig.1. The plot of the remaining activity against the no. mol PLP bound/mol enzyme of thermophilic isomerase. The insert: Effect of PLP-binding on the activity in the absence (△) and presence of 2 mM G6P (•) or 10 mM P_i (○). The enzyme was incubated with 720 mol PLP/mol enzyme for the time indicated in the figure and then reduced with borohydride.

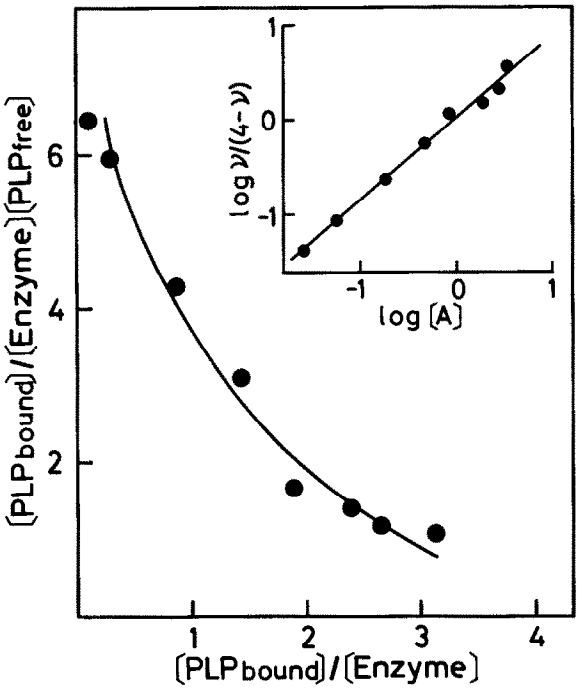

Fig.2. Scatchard plot for the PLP-binding to thermophilic isomerase. Insert: Hill plot for PLP-binding. [A], concentration of free PLP; ν, the no. mol PLP bound/mol enzyme. The solid line is a theoretical curve obtained by the Hill equation [9], $\log \nu/(4-\nu) = c\log[A] - c\log K$, in which $c = 0.82$ and $K = 1.3$ mM.

sites which are equivalent and independent [1]. The plot of the remaining activity against the no. mol PLP bound/mol enzyme for the thermophilic isomerase, on the other hand, was not linear but concave (fig.1). Even when the enzyme was treated with 720 mol PLP/mol enzyme (maximum amount of PLP that could be dissolved in the modification mixture), the enzyme activity was not completely inhibited, and maximally 3.4 mol PLP were bound to 1 mol enzyme.

The inactivation of the enzyme by PLP was considerably protected by 2 mM G6P or 10 mM Pi, a competitive inhibitor [2] (fig.1). This result suggests that the inactivation of the enzyme on PLP treatment was due to the modification of the essential lysine residues by PLP.

As shown in fig.2, the Scatchard plot for PLP-binding to the enzyme also deviated from linearity and was concave. The enzyme consists of 4 identical subunits [2]. The results in fig. 1,2 may therefore indicate that the enzyme has 4 PLP-binding sites or 4 essential lysine residues (one for each subunit) and that the enzyme contains 2 sets of the PLP-binding sites of different dissociation constants, since the enzyme is a dimeric dimer [2], or that the PLP-binding sites exhibits negative cooperativity. The experimental data in fig.2 could not be fitted to any theoretical curve obtained by assuming any values for the two dissociation constants. We therefore concluded that there is negative cooperativity in PLP-binding to the enzyme, as in the case of NAD-binding to glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) [8]. The Hill coefficient for PLP-binding to the thermophilic isomerase was estimated to be 0.82 from the slope of the Hill plot shown in fig.2.

The thermophilic isomerase and the PLP-treated enzyme with 3.0 modified lysine residues/enzyme molecule exhibited an emission maximum at 314 nm, probably due to tryptophan residues, when excited at 285 nm. The fluorescence intensity at 314 nm of the treated enzyme, however, was 78% that of the untreated enzyme. The modified enzyme exhibited another emission maximum at 390 nm, probably due to the phosphopyridoxyllysine residues. On excitation at 285 nm for 1 h, the intensity of the emission at

390 nm decreased to zero and the intensity at 314 nm increased to the same level as that of the unmodified enzyme. It has been reported that when glycogen phosphorylase *b* is excited at 280 nm energy transfer between tryptophan and phosphopyridoxyllysine residues may occur and phosphopyridoxyllysine residues are gradually decomposed on prolonged excitation [10]. The results obtained with the thermophilic isomerase may therefore indicate that tryptophan residues are located near the PLP-binding site (lysine residues) in the enzyme.

The results on the PLP-binding property of the thermophilic isomerase suggest a possible negative cooperativity in substrate-binding and therefore in enzyme activity, although the Lineweaver-Burk plot for the enzyme was shown to be linear [2]. Careful determination of the plots at 65°C by colorimetric assay and at 30°C by coupled assay showed that all the plots deviated from linearity and were concave (fig.3). The enzymes used in the coupled assay of the forward or backward reaction (phosphofructokinase, pyruvate kinase and lactate dehydrogenase, or phosphoglucose dehydrogenase) have never been reported to exhibit cooperativity. The results shown in fig.3 therefore may indicate negative cooperativity in the activity of the thermophilic isomerase in both forward and the backward directions. The Hill coefficients for the forward and backward reactions at 30°C and that for the forward reaction at 65°C were estimated to be 0.73, 0.70 and 0.86, respectively, from the Hill equation [11]. The Hill coefficients for the backward reactions at 30°C of the PLP-treated enzymes with 1.1, 2.2 and 3.0 modified lysine residues/molecule of enzyme were 0.71, 0.80 and 0.95, respectively. The results support negative cooperativity in the activity of the thermophilic isomerase.

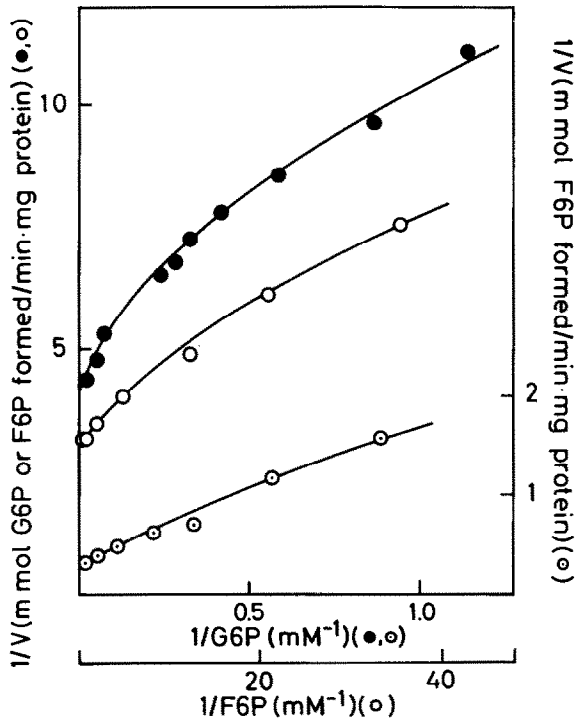

Fig.3. Lineweaver-Burk plots for the forward (●) and backward (○) reactions at 30°C and for the forward reaction at 65°C (⊙). The solid lines are the theoretical curves obtained by the Hill equation [11], $\log v/(V_{max} - v) = c\log [S] - c\log K$, in which $c = 0.73$ and 0.70 and $K = 1.4$ and 0.05 mM for the forward and backward reactions at 30°C, respectively, and $c = 0.86$ and $K = 4.4$ mM for the forward reaction at 65°C.

Phosphoglucose isomerase isolated from various living organisms have never been reported to exhibit cooperativity [12]. This paper is therefore the first demonstration of the isomerase exhibiting negative cooperativity in both enzyme activity and also in its PLP-binding property. The intracellular concentrations of G6P and F6P in actively growing cells were determined to be 0.34 and 0.04 mM, respectively, which lie in the substrate concentration ranges over which the kinetics were measured (fig.3). This fact indicates that negative cooperativity in enzyme reaction of the isomerase may actually operate in the organism.

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