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SUBSTRATE-INDUCED INTRAMOLECULAR PROTON TRANSFER IN 6-PHOSPHOGLUCONATE DEHYDROGENASE FROM *CANDIDA UTILIS*

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Formation of binary complex between 6-phosphogluconate dehydrogenase (6-phospho-D-gluconate:$NADP^+$ 2-oxidoreductase (decarboxylating), EC 1.1.1.44) from *Candida utilis* and 6-phosphogluconate was investigated by means of ultraviolet difference spectroscopy. The formation of the enzyme-substrate complex induces in the difference spectrum a positive peak the wavelength and extinction coefficient of which agree well with a tyrosine ionization. Titrimetric studies indicate that the formation of the binary complex is not coupled to a proton release from the protein. These data support an intramolecular proton transfer from a tyrosine to other functional group. This proton transfer could be correlated to the conformational change induced by substrate in 6-phosphogluconate dehydrogenase.

Formation of a binary complex between 6-phosphogluconate dehydrogenase from *Candida utilis* and its substrate, 6-phosphogluconate, results in an extensive modification of the properties of the enzyme. Compared to free enzyme, the enzyme-substrate complex shows lower reactivity toward several chemical [1] and denaturating agents [2], different binding stoichiometry with analogues of $NADP^+$ (Ref. 3 and Dallocchio, F., Matteuzzi, M. and Bellini, T., unpublished data), and altered dissociation constants with coenzymes and coenzyme analogues. These data clearly indicate that the binding of the substrate to the 6-phosphogluconate dehydrogenase induces a conformational change in the protein.

We have studied the modification of the absorption spectrum of the enzyme upon binding of 6-phosphogluconate. The difference spectrum shows two positive peaks, at 294 and 239 nm (Fig. 1); this spectrum resembles the tyrosine ionization difference spectrum. The extent of the extinction difference, induced by the formation of the binary complex, is function of the substrate concentration. Plotting the reciprocal of the millimolar extinction difference at 294 nm against the reciprocal of the substrate concentration, a straight line is obtained (Fig. 1, insert), which allows us to calculate a K_{diss} of 100 μM for the 6-phosphogluconate, and a maximum value for $\Delta\epsilon = 2.34\ mM^{-1} \cdot cm^{-1}$. This last value well agrees with the reported $\Delta\epsilon$ at 295 nm for the tyrosine ionization [4].

At short wavelengths the enzyme difference spectrum shows a less stringent correspondence with the spectrum of the tyrosine ionization. However, many amino acid residues absorb in the 240 nm region, and furthermore it is well known that free amino acids are not correct standards for the spectroscopic behaviour of the proteins.

Thus, the more likely interpretation of the difference spectrum induced by the substrate, is that a tyrosine residue of the enzyme ionizes upon binding of the 6-phosphogluconate. A similar tyrosine ionization has been reported in the formation of ternary

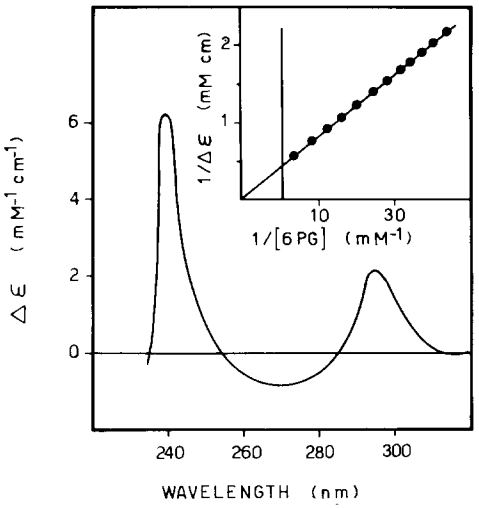

Fig. 1. Difference spectrum of 6-phosphogluconate dehydrogenase-substrate complex vs. 6-phosphogluconate dehydrogenase. The enzyme (11 μM in subunits). and the substrate (2 mM), dissolved in 50 mM triethanolamine buffer, pH 7.5, were placed in two compartment cuvettes; after recording the baseline, the sample cuvette was carefully mixed and the spectrum recorded. Insert: double-reciprocal plot of dependence of the millimolar extinction difference at 294 nm from the substrate concentrations. The points were obtained from a set of difference spectra recorded at different concentrations of 6-phosphogluconate.

complexes of alcohol dehydrogenase [5].

The proton released from the tyrosine could be transferred to the medium or to other amino acid residues of the protein. In the first case the formation of the binary complex should be coupled to a decrease of the pH of the solution. A direct measurement of the protons released in the medium, during the formation of the binary complex, has been accomplished with an apparatus as described by Lewis et al. [6]. When 6-phosphogluconate was added to 1 ml of 100 μM enzyme solution, in 0.1 mM phosphate buffer, pH 7.5 (final concentration 2 mM), the pH of the reaction mixture was unchanged. Additions of a few μl of 5 mM HCl and back titration with 5 mM KOH indicate that the sensitivity of the apparatus allows us to appreciate a proton release of as little as 5% of the protein concentration, ruling out that the proton release could be masked by the buffering properties of the solution. This means that the formation of the enzyme-substrate complex occurs without production, or consumption, of free hydrogen ions. Thus, the proton released from the tyrosine is transferred to another amino-acid residue of the enzyme, or to the bound substrate.

The data reported here clearly indicate that the formation of a binary complex between 6-phosphogluconate dehydrogenase and 6-phosphogluconate causes the ionization of a tyrosine residue and an intramolecular proton transfer. We have previously reported that the binding of the substrate to 6-phosphogluconate dehydrogenase induces a conformational change [3], thus it is likely that the intramolecular proton transfer could be directly correlated to the conformational change induced by the binary complex formation.

The ionization of a tyrosine residue at pH 7.5 is unlikely on the basis of a simple change of the exposure to the solvent. A more likely interpretation is that the conformational change is correlated to a modification of the ionization state of the protein.

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