1-ANILINO-8-NAPHTHALENE SULFONATE BINDING AS A PROBE FOR SUBSTRATE MEDIATED CHANGE IN CONFORMATION OF TRANSALDOLASE

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1. Introduction

Transaldolase (D-sedoheptulose-7-phosphate: D-glyceraldehyde-3-phosphate dihydroxyacetone transferase, E.C. 2.2.1.2) has been shown [1, 2] to catalyze the transfer of "active dihydroxyacetone" in the following reversible reaction:

fructose-6-P + erythrose-4-P

⇌ sedoheptulose-7-P + glyceraldehyde-3-P.

The enzyme crystallized from *Candida utilis* has a molecular weight of 66,000 and is composed of 2 identical subunits [3]. In the absence of the acceptor erythrose-4-phosphate, a stable enzymatically active intermediate accumulates which has been identified as a Schiff base containing dihydroxyacetone linked to the ε-amino group of a lysine residue at the active site of the enzyme [4, 5]. Dealdolization of the substrate, fructose-6-phosphate, involves the loss of a proton from the C-4 hydroxyl group. Recently we reported experimental results which suggest that the imidazole group of a histidine residue acts as a base catalyst, removing this proton and promoting the aldol cleavage reaction [6].

Photooxidation experiments further revealed that this critical histidine residue is more resistant to dye sensitized photooxidation in the transaldolase dihydroxyacetone complex than it is in the native enzyme. Histidine in the complex might be protected against photooxidation either by protonization or by substrate induced conformational change which makes histidine less accessible to the dye Rose Bengal.

In order to obtain more information about the nature of the active site and the mechanism of complex formation, binding studies were carried out using 1-anilino-8-naphthalene sulfonate (ANS) as a fluorescent probe for conformational states. The usefulness of ANS as a fluorescent probe of non-polar binding-sites in studies with enzymes and proteins has been demonstrated by a number of investigators [7–10].

2. Experimental methods

2.1. Materials

Transaldolase (type III) was purified and crystallized from *Candida utilis* by the procedure of Pontremoli et al. [11] as modified by Tsolas [3]. The enzyme was dialyzed overnight in sodium phosphate buffer 0.05 M, pH 7.6. The specific activity was 80 to 95 I.U./mg of protein. Crystalline α-glycerophosphate dehydrogenase, triosephosphate isomerase, phosphoglucose isomerase, glucose-6-phosphate dehydrogenase and the sodium salts of fructose-6-phosphate and NADH were obtained from Boehringer Mannheim Corp. D-Erythrose-4-phosphate was prepared from glucose-6-phosphate according to the method of Baxter et al. [12]. The ammonium salt of 1-anilino-8-naphthalene sulfonic acid was obtained from EGA Chemie KG, Steinheim, West Germany. The latter was twice recrystallized from hot water.

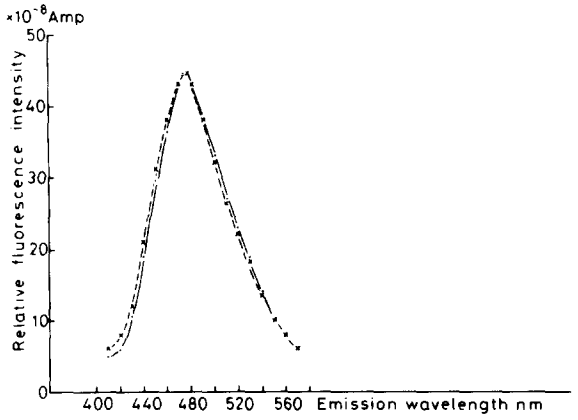

Fig. 1. Fluorescence emission spectra of ANS bound to transaldolase in the absence (. – .) and presence (x – – x) of substrate fructose-6-phosphate.

Conditions: transaldolase 2×10^{-5} M; ANS 2×10^{-3} M; fructose-6-phosphate 2×10^{-3} M; test volume : 2 ml; excitation at a wavelength of 366 nm. The scale of the relative fluorescence intensity is normalized in order to compare the emission maxima for both measurements.

The pale green crystals were dried at 120° for several hours.

2.2. *Analytical procedures*

Transaldolase was assayed as described by Tsolas and Horecker [13]. Protein concentrations were determined by the method of Bücher [14]. The factor used was 0.33 mg per absorbance unit at 340. The molar concentration of transaldolase was calculated from the protein determination, based on a molecular weight of 66,000.

Routine fluorescence measurements were performed at room temperature in an Eppendorf spectrophotofluorometer using the 313 + 366 nm filter for excitation and the 420 – 3000 nm filter for measuring the fluorescence emission. All fluorescence experiments were corrected for fluorescence of dye, enzyme and other reagents when added. Emission spectra were taken in collaboration with Dr. M.Boldt (Max-Planck-Institut für Arbeitsphysiologie Dortmund, Germany) with a spectrofluorometer of personal design and corrected for photomultiplier and monochromator characteristics.

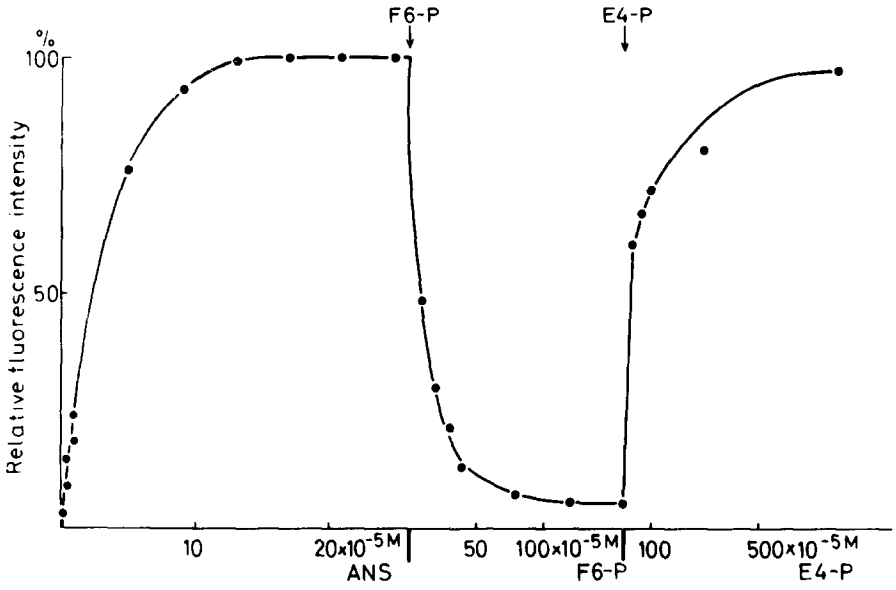

Fig. 2. Effect of substrate fructose-6-phosphate and erythrose-4-phosphate on transaldolase-ANS fluorescence. Experiments performed in 0.05 M sodium phosphate buffer, pH 7.6 with a transaldolase concentration of 7.7×10^{-6} M; test volume: 3 ml.

2.3. *Determination of n and K*

The number of dye-binding sites per mole of transaldolase and the dissociation constant for the complex (K_D) were determined for the ANS-enzyme interaction according to the general method developed by Klotz et al. [15] which is described in detail by Weber and Young [7].

3. Results

Addition of transaldolase to a solution of ANS in 0.05 M soldium phosphate buffer, pH 7.6 resulted in a large enhancement of the fluorescence intensity of the dye. As shown in fig. 1, the emission maximum was at a wavelength of 476 nm and did not change when the substrate fructose-6-phosphate was present. As expected, the binding of ANS to the enzyme causes a shift of the emission maximum to a shorter wavelength. The emission maximum of an ANS solution in 0.05 M sodium phosphate buffer was at 510 nm.

Addition of increments of ANS to a constant transaldolase concentration produced a progressive increase in fluorescence as shown in fig. 2. The fluorescence intensity reaches a saturation level at an ANS concentration of about 1.2×10^{-4} M. In a series of experiments in which different concentrations of ANS and enzyme were used, a dissociation constant (K_D) of 2.2×10^{-5} M for the transaldolase-ANS complex was determined. The number for ANS binding sites per transaldolase molecule calculated according to Klotz et al. [15] was found to be 2.2 (± 0.2). Each subunit therefore binds one dye molecule with a K_D of 2.2×10^{-5} M. As shown in fig. 2, addition of increments of substrate fructose-6-phosphate to the transaldolase-ANS complex causes an almost complete disappearance of fluorescence, indicating an influence of substrate on the binding sites for ANS. In control experiments, we have found that other hexose-phosphates such as fructose-1,6-diphosphate and glucose-6-phosphate have no effect on the fluorescence intensity of the transaldolase-ANS complex. Addition of the second substrate, erythrose-4-phosphate, causes an increase of fluorescence intensity to the original level, which is expected from the reaction mechanism (see discussion).

On fixation of the triose to the enzyme by reduction of the Schiff base with sodium borohydride, no effect of ANS on the fluorescence intensity was observed indicating that the reduced complex does not bind ANS.

Stop-flow experiments were carried out to study the kinetics of ANS binding to transaldolase as well as its displacement by fructose-6-phosphate. It was found that both reactions were completed within the time limit of the stop-flow apparatus of 15 msec.

4. Discussion

The experiments demonstrate that transaldolase, like other proteins, has a limited number of binding sites for the fluorescent dye, ANS and greatly enhances its fluorescence. The number of dye-binding sites which has been determined to be 2, is in good agreement with the concept of a dimer structure of transaldolase obtained by hybridization experiments with two isoenzymes of *Candida* transaldolase [3]. The fact that fructose-6-phosphate causes an almost complete disappearance of fluorescence indicates that the non-polar binding site of the dye is influenced by substrate binding probably due to a conformational change. The dissociation constant for the transaldolase-ANS complex was 2.2×10^{-5} M. The Michaelis constant for fructose-6-phosphate has been reported to be 3.4×10^{-4} M [16]. Since a 3 to 4-fold excess of fructose-6-phosphate which has a ten-times lower affinity displaced ANS completely from the enzyme, a conformational change around the active site induced by substrate leading to a loss of hydrophobicity has to be assumed. From earlier experiments [4–6] we know that transaldolase has only one active site for fructose-6-phosphate. The concept of a substrate mediated conformational change occurring around the active site could also explain the complete displacement of two moles of ANS by the binding of only one mole of dihydroxyacetone. It should be assumed that ANS is bound separately on the two monomers which contribute to one active site. This explanation implies a substrate action on the ANS binding sites on both monomers. Native transaldolase contained two histidine residues [18].

From photooxidation experiments [6], we know that the loss of one histidine residue abolishes not

only overall catalytic activity, but also the ability of the enzyme to form the Schiff base. The transaldolase dihydroxyacetone complex is less sensitive to photoinactivation than the native enzyme indicating that the histidine residues in the complex were protected from photooxidation. This result may also support the assumption of a conformational change of the active site which makes histidine less accessible than in the native transaldolase. On the other hand, protection of the histidine residues in the complex from photooxidation could also be explained by assuming that they were present in the protonated state even at pH 7.6.

Addition of the second substrate, erythrose-4-phosphate, to the mixture containing transaldolase, ANS and fructose-6-phosphate causes an increase of fluorescence intensity to the original level. In the presence of erythrose-4-phosphate dihydroxyacetone of the complex is transferred to this acceptor forming sedoheptulose-7-phosphate and releasing native transaldolase which binds ANS again. The change in the fluorescence intensity of the transaldolase-ANS complex by fructose-6-phosphate and erythrose-4-phosphate strongly indicates that the interaction between ANS and the substrate is specific. From these data it is very likely that ANS is bound close to the active site of transaldolase. The binding of fructose-6-phosphate apparently causes a conformational change affecting also the binding sites of ANS and leading to a loss of hydrophobicity. Therefore ANS is released from the enzyme as indicated by a decrease of fluorescence intensity. From the stop-flow experiments it can be concluded that the substrate mediated transient between the two conformational states in transaldolase is faster than 15 msec.

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