

Binding of the Fluorescent Dye 8-Anilino-naphthalene 1-Sulfonic Acid to the Native and Pressure Dissociated β_2 -Dimer of Tryptophan Synthase from *Escherichia coli*

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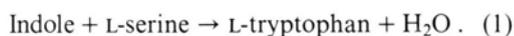
The β_2 -dimer of tryptophan synthase from *Escherichia coli* exhibits weak binding of 8-anilino-naphthalene-1-sulfonic acid (ANS). Titrating the dye at 0.2 mM concentration with the apo- β_2 -dimer at atmospheric pressure causes increased fluorescence emission at 480 nm ($\lambda_{exc} = 380$ nm), corresponding to unspecific binding of the ligand to hydrophobic residues.

Increasing hydrostatic pressure affects ANS binding. Up to 700 bar, a sigmoidal increase of ANS fluorescence reflects an increase in hydrophobic surface area, probably caused by subunit dissociation. At ~ 1 kbar, a maximum is reached; beyond this value, pressure competes with ligand binding causing fluorescence emission to be decreased again.

Pressure release leads to a drastic fluorescence enhancement, ascribed to ANS binding to the partially and reversibly denatured enzyme. Plotting the total fluorescence enhancement vs. pressure yields a profile which parallels the pressure dependent dimer \rightleftharpoons monomer transition monitored by subunit hybridization (T. Seifert, P. Bartholmes, and R. Jaenicke, *Biochemistry*, in press).

Introduction

Tryptophan synthase (L-serine hydro-lyase, adding indoleglycerolphosphate, EC 4.2.1.20) from *Escherichia coli* is an $\alpha_2\beta_2$ holoenzyme complex. Its isolated β_2 -dimer catalyzes the final step in tryptophan biosynthesis



It is a stable entity which can be dissociated into its constituent polypeptide chains only under strongly denaturing conditions [1]. Indirect evidence from previous denaturation and hybridization studies [2] indicated that high hydrostatic pressure causes deactivation, paralleled by subunit dissociation. Reactivation was shown to be governed by consecutive first-order processes, thus suggesting that

Enzyme: Tryptophan synthase (L-serine hydro-lyase, adding indoleglycerolphosphate, EC 4.2.1.20).

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subunit assembly is a fast reaction following the rate-determining formation of the "structured monomer" [3].

In the present study pressure dependent binding of the fluorescent dye 8-anilino-naphthalene-1-sulfonic acid (ANS) was applied to investigate the involvement of hydrophobic interactions in the subunit contacts of the β_2 -dimer of tryptophan synthase [4–6].

Materials and Methods

Tryptophan synthase β_2 -dimers were purified from the A2/F'A2 mutant strain of *E. coli* and stored at -76°C in 0.6 M potassium phosphate buffer pH 7.8, containing 0.2 mM dithioerythritol (DTE), 5 mM EDTA and 0.2 mM pyridoxal-5'-phosphate (PLP) [7].

Buffer substances and EDTA were purchased from Merck (Darmstadt), ANS (Mg-salt) and PLP from Serva (Heidelberg), DTE from Roth (Karlsruhe). Quartz-bidistilled water was used throughout. 0.1 M triethanolamine \cdot HCl, 0.1 M NaCl, 2 mM DTE, 0.5 mM EDTA, pH 7.8 (10°C) was used as standard buffer.

The PLP-free enzyme (apo β_2) was prepared by passing holoenzyme through a small band of 0.5 M hydroxylammonium chloride on a Sephadex G-25 column (0.5×25 cm), equilibrated with standard buffer. Enzyme concentrations were determined spectrophotometrically in 0.1 N NaOH: $A_{290\text{ nm}}^{0.1\%} = 0.75 \text{ cm}^2 \cdot \text{mg}^{-1}$ [7].

Absorbance and fluorescence spectra were measured using Zeiss DMR 10 and Hitachi-Perkin Elmer MPF 44A spectrometers with temperature jacketed cuvette holders (10°C). The fluorescence emission at elevated pressure was measured as described previously [8].

Results and Discussion

As shown in Fig. 1, the apo β_2 -dimer of tryptophan synthase shows maximum fluorescence at 317 nm ($\lambda_{exc} = 280$ nm). Adding 0.2 mM ANS, the intrinsic protein fluorescence is quenched while the emission spectrum of the bound ligand emerges. Direct excitation of ANS at 380 nm yields two emission maxima of equal height at 462 and 480 nm;



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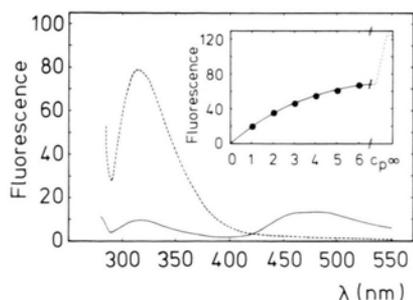


Fig. 1. Fluorescence emission spectra of tryptophan synthase β_2 -dimer in the absence and in the presence of ANS ($\lambda_{\text{exc}} = 280$ nm). Standard buffer, $c_p = 0.1$ mg/ml, 10°C . Broken line: apo β_2 -dimer; solid line: apo β_2 -dimer plus $200\ \mu\text{M}$ ANS. *Insert:* Fluorescence titration of $200\ \mu\text{M}$ ANS with apo β_2 tryptophan synthase ($\lambda_{\text{exc}} = 380$ nm, $\lambda_{\text{em}} = 480$ nm). Final value taken from the extrapolation of the Eadie-Hofstee plot given in Fig. 2.

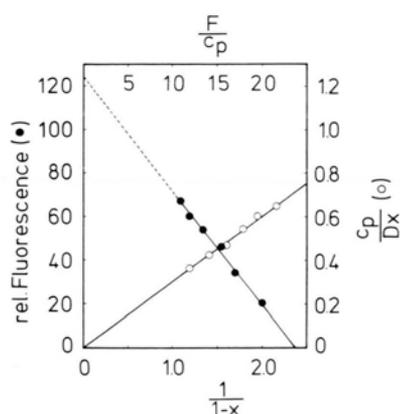


Fig. 2. Determination of the maximum fluorescence (cf. Fig. 1, insert), and the stoichiometry and dissociation constant of ANS binding to tryptophan synthase β_2 -dimer at 1 bar. (●) Eadie-Hofstee plot according to

$$F = -K_M \frac{F}{c_p} + F_{\text{max}} ;$$

(○) linearization according to Eqn. (2).

with free ANS no significant fluorescence emission is detectable. These observations indicate weak binding of the fluorophore to the native enzyme. To determine the stoichiometry a $0.2\ \text{mM}$ solution of ANS was titrated with the apo β_2 subunit, monitoring the relative fluorescence emission at $480\ \text{nm}$ (insert, Fig. 1). The usual ANS titration of the protein could not be applied because the ligand quenches its own fluorescence at high concentrations so that the ANS concentration must be kept constant (and sufficiently low).

Because of the low binding constant an Eadie-Hofstee plot (Fig. 2) was used in order to estimate the saturation value F_{max} . As taken from the linear relationship, using the *total* instead of the *free* protein concentration does not cause a significant error. Linearization of the binding data according to Eqn. (2) ([9], cf. Fig. 2)

$$\frac{c_p}{Dx} = \frac{1}{N} + \frac{K}{ND(1-x)} \quad (2)$$

with c_p = total protein concentration, D = total ANS concentration, x = relative proportion of bound ANS taken from rel. fluorescence (F/F_{max}), N = moles ANS per mole β_2 and K = dissociation constant of the ANS complex [11], yields a low value for the intercept $c_p/Dx = 1/N$ (for $\frac{1}{1-x} = 0$), corresponding to $N \geq 10$ binding sites. Introducing this number into the slope $m = K/ND$ of a c_p/Dx vs $\frac{1}{1-x}$ plot (Fig. 2), allows us to estimate the equilibrium constant: $K \geq 0.6\ \text{mM}$. The Hill coefficient is found to be $n = 1.0 \pm 0.1$.

Summarizing the results at atmospheric pressure, the apo β_2 -dimer is found to show extensive non-cooperative ANS binding. In contrast to a number of other proteins, the ligand exhibits only low affinity.

In general, ANS binding is applied to monitor the accessibility of hydrophobic regions of a given molecule [19, 20]. However, it should be mentioned that the ligand may undergo similar spectral changes in the presence of high concentrations of MgCl_2 [12]. Irrespective the explanation of this effect in terms of an interconversion of two conformations of the dye, the formation of an intramolecular hydrogen bond in the course of the attachment of ANS to hydrophobic surface areas is generally considered to account for the observed fluorescence enhancement.

At elevated pressure, the enzyme exhibits complex behaviour. The time course of a representative experiment is given in Fig. 3, the pressure dependence over the whole pressure range in Fig. 4. Both figures suggest the superposition of two antagonistic effects causing a decrease in ANS fluorescence on one hand (e.g. phase A and D in Fig. 3), and an increase on the other (phase B and C). According to the previously mentioned explanation of the fluo-

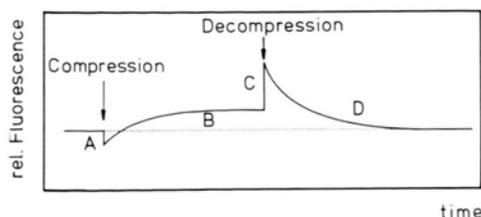


Fig. 3. Schematic profile of the pressure dependent fluorescence emission of ANS in the presence of tryptophan synthase β_2 -dimer at 1000 bar ($\lambda_{exc} = 380$ nm, $\lambda_{em} = 480$ nm). $c_p = 0.1$ mg/ml; $c_{ANS} = 200$ μ M.

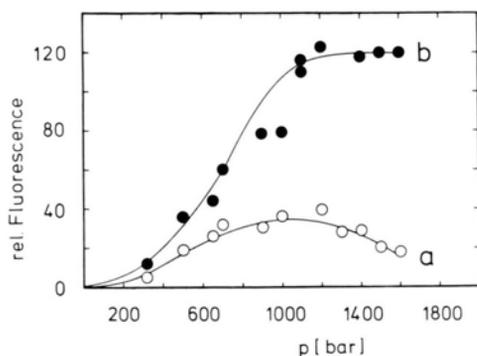


Fig. 4. Pressure dependence of the change in ANS fluorescence depicted in Fig. 3 ($\lambda_{exc} = 380$ nm; $\lambda_{em} = 480$ nm). Standard buffer, 10 °C. a. Increase in ANS fluorescence under pressure (phase B, Fig. 3). b. Total increase in ANS fluorescence (phase B plus C, Fig. 3).

rescence enhancement, the initial fast decrease in ANS fluorescence (phase A in Fig. 3) may be attributed to the pressure-induced desorption of ANS molecules, weakly bound to the protein surface. The vanishing ANS fluorescence at high pressures (Fig. 4, curve A), illustrates a similar dissociation effect for ANS binding sites with higher affinity.

Phase B may be attributed to the exposure of hydrophobic binding sites during the slow reversible denaturation (dissociation) of the β_2 -dimer. Obviously, hydrophobic areas differ in their affinity towards ANS so that after a plateau value has been reached in phase B, pressure release generates an "overshoot" effect (phase C). This may be easily explained by the assumption that a great number of additional weak binding sites on the dissociated protein is saturated at atmospheric pressure; upon renaturation (reassociation) the initial state is recovered so that hydrophobic sites exposed at high pressure are buried again in the interior of the

native molecule. Phase D represents the slow re-shuffling reaction limiting the regain of native protein fluorescence, as well as enzyme activity [2].

Adding the amplitudes of phases B and C at varying pressure allows us to calculate the total ANS binding in the process of the pressure induced reversible denaturation (dissociation) (Fig. 4, curve B). The profile confirms the dissociation profile determined previously on the basis of high pressure deactivation and subsequent hybridization with β -monomers chemically modified by sodium borohydride reduction [2, 13].

Conclusions

High hydrostatic pressure in the biologically relevant pressure range [14] affects the conformation, as well as the state of association of oligomeric proteins without perturbing the covalent backbone structure of the constituent polypeptide chains. Regarding non-covalent interactions, the most important candidates involved in pressure effects are hydrophobic and electrostatic interactions [14, 15]. An increase in hydrophobic surface area is expected to promote "clathrate formation" in the aqueous solvent. The sign and absolute value of the reaction volume of the process is still controversial. The reason is that the net effect is the sum of many contributions which may not compensate each other [16–18].

In the case of lactate dehydrogenase, no significant ANS binding to neither the native, nor the pressure dissociated enzyme has been observed; fluorescence of the dye is obtained only after denaturation, e.g. at acid pH [16]. The present experiments show that the β_2 -dimer of tryptophan synthase exhibits a quite different behaviour. Numerous binding sites on the native enzyme are affected by pressure in a subtle way, indicating a variety of binding sites with different affinities. Varying pressure shifts the respective binding equilibria so that denaturation (dissociation), as well as renaturation (reassociation) can be monitored. The results corroborate independent reconstitution studies, applying subunit hybridization [2, 13].

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