High-pressure dissociation of the β_2-dimer of tryptophan synthase from *Escherichia coli* monitored by sucrose gradient centrifugation*

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The isolated β_2-dimer of *Escherichia coli* tryptophan synthase exhibits reversible high-pressure deactivation and hybridization with an equilibrium transition at 690 and 870 bar for the apoenzyme and holoenzyme, respectively. To investigate the hypothetical dissociation mechanism ultracentrifugal analysis has been applied. In a conventional swing-out rotor (r_{max} = 16 cm, fill-height 9 cm) a pressure gradient of $1 < p <$ 1840 bar is formed at maximum speed (40000 rpm). Using a sucrose gradient to stabilize the particle distribution, pressure-dependent alterations of the state of association of oligomeric systems may be determined. In the present experiments ovalbumin (with a molecular mass close to the β-monomer) has been used as a reference. The radial sedimentation velocity of the β_2-dimer (in 5–20% sucrose, 10°C) is found to decrease significantly at $p \cong 850$ bar. From the slopes in an $r-r_o$ *vs* t plot the limiting values for the particle weight at the meniscus and the bottom of the tube are found to be the β_2-dimer (M_r = 85800) and the β-monomer (M_r = 42900), thus proving pressure-dependent dissociation. Since sucrose stabilizes the native quaternary structure, the $\beta_2 \longrightarrow 2\beta$ transition is shifted towards higher pressures compared to the dissociation in standard buffer. Conventional quench experiments in high-pressure cells in the presence of 13% (w/v) sucrose confirm the result of the sucrose gradient centrifugation with respect to the critical pressure where deactivation (and dissociation) occur.

High-pressure dissociation *Sucrose gradient centrifugation* *Ultracentrifugation* *Tryptophan synthase*

1. INTRODUCTION

The isolated β_2-dimer of *Escherichia coli* tryptophan synthase has been shown to exhibit reversible high-pressure deactivation [1,2]. Hybridization experiments using the native enzyme and chemically modified (reduced) holo β-chains suggest pressure-induced dissociation to be the underlying mechanism [1]. However, this evidence is not free from ambiguity because deactivation and hybridization may equally reflect conformational changes and subunit exchange by way of encounter complexes [3]. In the case of other oligomeric enzymes the hypothetical dissociation mechanism was unequivocally proved by rate-determining second-order steps in the process of reconstitution after pressure release [4–6]. As indicated by previous reconstitution studies after dissociation of the β_2-dimer in 4.5 M guanidine·HCl, association steps are not rate-determining in the present case. Instead, reactivation kinetics suggest a unimolecular 'reshuffling' process to be rate-limiting whereas dimerization is found to be close to diffusion controlled [1,7]. Thus independent experimental methods are required to unravel the mechanism underlying the observed high pressure deactivation.

Ultracentrifugal analysis allows us to determine

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alterations of the state of association with varying hydrostatic pressure. Using conventional rotors, pressures up to 2 kbar may be generated. Considering then the radial velocity of a given assembly system as a function of the distance from the meniscus, the actual particle size may be directly correlated with the hydrostatic pressure at a given radius. Making use of this approach (applying a stabilizing sucrose gradient) provides clear evidence that the β_2-dimer of tryptophan synthase undergoes pressure-dependent dissociation. The $\beta_2 \rightleftharpoons 2\beta$ transition occurs in the same pressure range where deactivation and hybridization take place.

2. 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 as in [8]. Buffer substances, EDTA, and sucrose were purchased from Merck (Darmstadt), pyridoxal 5′-phosphate (PLP) and ovalbumin from Serva (Heidelberg), and dithioerythritol (DTE) from Roth (Karlsruhe). Quartz-bidistilled water was used throughout. 0.1 M triethanoleamine·HCl, 0.1 M NaCl, 2 mM DTE, 0.5 mM EDTA, pH 7.8 (10°C) was used as standard buffer; since deprotonation of the buffer cation does not create additional charges, the pressure effect on the pH is expected to be negligible [9]. PLP-free apo β_2-dimer was prepared as in [1]. Enzyme concentration and specific activity were measured in a Zeiss DMR 10 spectrophotometer according to [10,11]: $A^{0.1\%}_{290nm}$ = 0.75 $cm^2 \cdot mg^{-1}$, A_{sp} = 8 IU·mg^{-1} (10°C). High-pressure quench experiments made use of autoclaves and a pressure-generating system described in [12]. After pressure release the enzyme solution was immediately removed, and the catalytic activity measured under normal pressure (25°C). To prevent reactivation during the test, 10 μg trypsin per ml of test medium were added [1].

Density-gradient centrifugation made use of a Hitachi 65P ultracentrifuge equipped with an RPS 40 T swing-out rotor (radial distances r_o and r_{max} of the meniscus and bottom: 7 and 16 cm, respectively). The pressure gradient along the tube depends on the given dimensions according to

$$p(r) = p(r_o) + \frac{1}{2}\varrho\omega^2 (r^2 - r_o^2) \quad (1)$$

Ignoring the density gradient, maximum speed (40000 rpm) yields a pressure range from 1 bar at the meniscus up to 1840 bar at the bottom. Pressure-dependent dissociation could be easily detected by a decrease in sedimentation velocity of the protein layered on top of the preformed sucrose gradient.

Analysis after varying sedimentation time made use of enzyme activity or UV absorption; 0.6 ml fractions were collected using a capillary and a peristaltic pump. Estimates of the molecular masses (M) were obtained from the radial positions (r) or from the corresponding sedimentation velocities (v) according to [13]

$$\frac{r_1}{r_2} = \frac{v_1}{v_2} = \left(\frac{M_1}{M_2}\right)^{2/3} \quad (2)$$

3. RESULTS AND DISCUSSION

As shown by previous quench experiments monitoring the residual activity immediately after pressure release, the cofactor, PLP, causes significant stabilization of tryptophan synthase towards pressure deactivation [2]. The midpoints of the equilibrium transition curves for the apo- and holoenzyme are 690 and 870 bar, respectively. To achieve sufficient resolution with regard to the expected change in molecular masses, ultracentrifugation was performed at maximum speed. Ovalbumin (with a molecular mass close to the size of the β-monomer) was chosen as a single-chain reference.

Applying 100 μl enzyme solution (5 mg/ml) on top of a 5–20% sucrose gradient, centrifugation time at 40000 rpm was varied from 12 to 60 h.

The result illustrated in fig.1 clearly proves the marker protein to show constant sedimentation velocity over the whole range of $r - r_o$. Tryptophan synthase exhibits anomalous sedimentation behavior with 'breaks' at 850 and 1300 bar for the apo- and holoenzyme, respectively. In the bottom part of the tube, the limiting slope of $(r - r_o)$ *vs* t tends to a value slightly below the velocity observed for ovalbumin (M_r = 45000); in the meniscus part, the slope is twice as high. The corresponding particle masses calculated according to eq.2 are in excellent agreement with molecular mass determinations of the β-chain and the β_2-dimer reported in the literature [7,14]. The broken

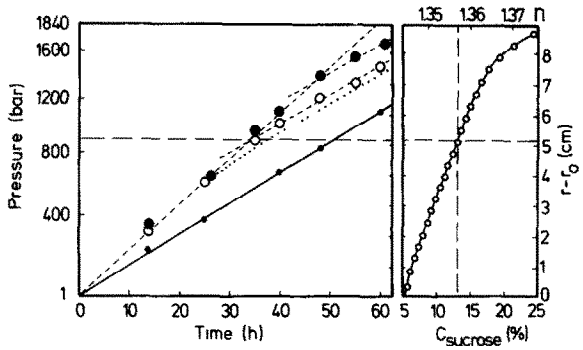

Fig.1. Sucrose gradient centrifugation of the β_2-dimer of tryptophan synthase and ovalbumin. (Left) Time dependence of sedimentation in the pressure gradient $1 < p < 1840$ bar; 40000 rpm, 5–20% sucrose in standard buffer (13 ml), 10°C. Centrifugation of 100-μl samples (5 mg/ml), layered on top of the gradient; determination of the position of the bands after indicated periods of time by monitoring enzymatic activity (tryptophan synthase) or absorption at 280 nm (ovalbumin) in 0.6 ml fractions. (●) Ovalbumin (M_r = 45000) as monomeric reference. (○, ●) Apo and holo β_2-subunits (40 μM PLP) of tryptophan synthase, respectively. (– – –) Sedimentation velocity of the β_2-dimer and the β-monomer, calculated according to eq.2 for M_r (β_2) = 85800 and M_r (β) = 42900, respectively. (···) Sedimentation profile of the apoenzyme calculated using the result of quench experiments in standard buffer, i.e., in the absence of sucrose (equilibrium transition at 690 bar). (Right) Refractometric analysis of the 5–20% sucrose gradient after 35 h centrifugation at 40000 rpm, 10°C. The equilibrium transition at 850 bar occurs at a sucrose concentration of 13.2 ± 0.2% (w/v).

lines in fig.1 give the theoretical slopes for M_β = 42900 and M_{β_2} = 85800.

To determine the midpoint of the $\beta_2 \rightleftharpoons 2\beta$ transition ($p_{1/2}$), hypothetical dissociation curves were transformed into the corresponding sedimentation profile, making use of $\nu_\beta = 0.63\ \nu_{\beta_2}$ (cf. eq.2) and $\nu(p) = [(100 - c)/100]M \cdot 0.37\ \nu_{\beta_2} + 0.63\ \nu_{\beta_2}$, with c_M = concentration of β-monomers given in % of total monomer concentration. In the latter equation, the pressure-dependent equilibrium ($c_M = f(p)$) is transformed into the average sedimentation rates of mixtures of β and β_2, with the limiting values for the meniscus ($c_M \longrightarrow 0$) $\nu(p) = \nu_{\beta_2}$, and for the bottom ($c_M \longrightarrow 100$) $\nu(p) = \nu_\beta$, respectively [2].

Applying the results of quench experiments (e.g., for the isolated apo β_2-dimer [2]: dissociation constant at atmospheric pressure, $K(p_o) = 4.5 \times 10^{-10}$ M, reaction volume, $\Delta V = -290$ ml/mol, and pressure where 50% dissociation occurs, $p_{1/2}$ = 690 bar), ultracentrifugation is found to exhibit systematic deviations (fig.1, dotted line): In the sedimentation experiment, the midpoint of the $\beta_2 \rightleftharpoons 2\beta$ transition is shifted to higher pressures (850 bar). The reason for this difference becomes clear if we consider the stabilizing effect of sucrose on proteins which is linked to the preferential hydration of the polar protein surface due to non-specific interactions [15–18].

As expected, this effect also holds for pressure-induced denaturation, dissociation and deactivation (inset, fig.2). Incubation of the β_2-dimer at varying sucrose concentrations and 850 bar, clearly demonstrates the stabilizing effect of the additive. Correspondingly, quench experiments in the presence of sucrose show a significant shift of the equilibrium transition towards higher pressures (fig.2). At the same time, the transition curve exhibits decreased cooperativity and a lower value of the reaction volume of the pressure-induced dissociation (ΔV = 180 cm^3/mol). The refractometric determination of the sucrose distribution along the tubes shows a slight non-linearity of the gradient (fig.1). The actual sucrose concentration at the midpoint of the equilibrium transition for

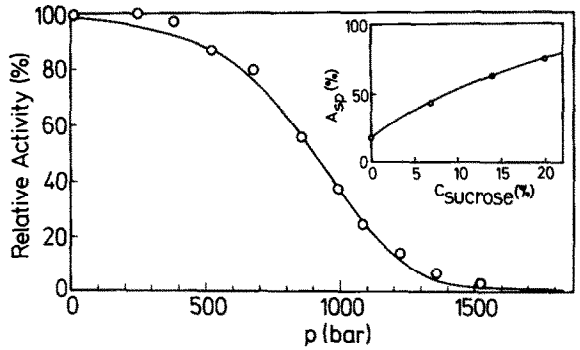

Fig.2. Pressure-dependent deactivation of the apo β_2-dimer of tryptophan synthase in the presence of 13% sucrose. Equilibrium values were determined after 3 h incubation at given pressures in standard buffer, 10°C. Residual activity was measured immediately after pressure release (<1 min). (Inset) Residual activity of the apo β_2-dimer of tryptophan synthase after 3 h incubation at 850 bar in the presence of varying sucrose concentrations; standard buffer, 10°C.

the apoenzyme is 13%. Comparing the results of the sucrose-gradient centrifugation to those obtained by quench experiments in an autoclave in the presence of 13% sucrose (inset, fig.2), a fair agreement regarding the equilibrium transition is achieved. Thus the present results provide unequivocal evidence that pressure-dependent deactivation of the β_2-dimer of tryptophan synthase is accompanied by subunit dissociation. As indicated by the high yield of reconstitution after pressure release, the dissociation is fully reversible. The recovered enzyme is indistinguishable regarding its enzymological and physico-chemical properties.

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