Influence of α-Subunits on the High-Pressure Stability of Apo and Holo β_2-Subunits in the Bienzyme Complex Tryptophan Synthase from *Escherichia coli*[†,‡]

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ABSTRACT: At a hydrostatic pressure of up to 2 kbar, the isolated α-subunit of tryptophan synthase from *Escherichia coli* proved to be a stable enzyme by virtue of specific activity as well as UV absorption and fluorescence emission spectra. The protein can therefore be regarded as a suitable effector for the investigation of structure−function relationships in the dimeric β_2-subunit under the influence of high hydrostatic pressure. Complete deactivation of the β_2-component in the $\alpha_2\beta_2$ bienzyme complex occurs above 1300 bar (midpoint of transition for $\alpha_2$apoβ_2, 790 bar; for $\alpha_2$holoβ_2, 1057 bar). Sucrose (13%) shifts both midpoints of transition to values higher by about 300 bar. As shown by sucrose gradient centrifugation and limited trypsinolysis, deactivation of the β_2-dimer is paralleled by dissociation into denatured β-chains. At 10 °C, the corresponding dissociation constants K at 1 bar as well as the reaction volumes of dissociation ΔV are calculated as 4.2×10^{-9} M and −196 mL/mol for the apo-β_2-component and as 9.8×10^{-19} M and −632 mL/mol for the holo-β_2-component in the bienzyme complex. Furthermore, large negative activation volumes are determined, reflecting the rate increase with increasing pressure: −89 mL/mol for the apo-β_2-dimer and −195 mL/mol for the holo-β_2-dimer. Pressure release after dissociation of the $\alpha_2\beta_2$ bienzyme complex into native α-subunits and completely deactivated β-protomers leads to 92% recovery of specific β_2 activity in the spontaneously reassociated apo bienzyme complex (with $t_{1/2}$ = 18 min) and to 78% in the holoenzyme (with $t_{1/2}$ = 27 min), respectively.

Various procedures have been used to dissociate protein complexes in order to understand subunit interaction. Among these were thermal transitions and pH changes along with different chemical agents such as urea, guanidinium salts, inorganic salts, thiols, detergents, and nonpolar organic solvents (Klotz et al., 1970; Reithel, 1963; Tanford 1968, 1970). The occurrence of pressure effects on associating protein systems in quench experiments (Jaenicke & Koberstein 1971, Jaenicke, 1981; Seifert et al., 1984a, 1985; Silva et al., 1986) and ultracentrifugation experiments (Creighton & Yanofsky, 1966; TenEyck & Kauzmann, 1967, Dicamelli et al., 1973; Seifert et al., 1984b) have been reported.

A system that is well-suited for studies of the factors involved in protein−protein association is the enzyme tryptophan synthase from *Escherichia coli*. The tetrameric αββα bienzyme complex can be regarded as an excellently characterized example for the influence of mutual, heterologous interactions between chemically different subunits on catalytic function and structural stability (Miles, 1979, 1991), and it catalyzes the following reactions:

$$\text{indoleglycerol 3-phosphate} + \text{L-serine} \xrightarrow{\alpha_2\beta_2} \text{D-glyceraldehyde 3-phosphate} + \text{L-tryptophan} + H_2O \quad (1)$$

$$\text{indoleglycerol 3-phosphate} \overset{\alpha}{\rightleftharpoons} \text{D-glyceraldehyde 3-phosphate} + \text{indole} \quad (2)$$

$$\text{indole} + \text{L-serine} \xrightarrow{\beta_2} \text{L-tryptophan} + H_2O \quad (3)$$

The α-subunit is a single polypeptide chain with a molecular weight of about 29 500 and a sedimentation coefficient $s^0_{20,w}$ of 2.7 S (Henning et al., 1962). The β_2-subunit exists as a tightly associated dimer of two identical polypeptide chains, with a molecular weight of about 43 000 each (Hathaway & Crawford, 1970), and a sedimentation coefficient $s^0_{20,w}$ of 5.1 S (Hathaway et al., 1969). The fully associated bienzyme complex has a $s^0_{20,w}$ value of 6.4 S (Creighton & Yanofsky, 1966, Goldberg & Creighton, 1966).

Even in the absence of the corresponding α-chains, the isolated β_2-subunit has been shown to be a remarkably stable dimer. It can be reversibly denatured and dissociated in the presence of 4.5 M guanidinum hydrochloride at acidic pH (Groha et al., 1978) or at high hydrostatic pressure (Seifert et al., 1982). Hybridization experiments (Seifert et al., 1985) and ultracentrifugal analysis (Seifert et al., 1984b) provided clear evidence that the β_2-dimer of tryptophan synthase undergoes high-pressure dissociation.

High pressure is a unique tool to obtain unperturbed thermodynamic and kinetic information characterizing denaturation and dissociation equilibria of enzymes. Compared to chemical denaturants, high reactivation yields are obtained since a comparatively high degree of structural information is conserved by applying hydrostatic pressure (Schade et al., 1980a,b). It has been repeatedly shown that pressures below 3 kbar do not affect the tertiary structure of single-chain

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proteins [for a review, see Heremans (1982), Weber and Drickamer (1983)], thus validating the use of pressures below this limit to promote the dissociation of oligomeric proteins into minimally altered subunits (Penniston, 1971; Schmid et al., 1979).

In the present study, we first examine the effect of pressure on the isolated α-subunit to prove its stability when separated from the corresponding β_2-subunit. Then, the role of the α-subunit as a positive effector of structural stability on the high-pressure denaturation and renaturation of the holo- and apo-β_2-subunit is defined by equilibrium and kinetic experiments.

Finally, we investigate pressure-dependent alterations of the state of association of the tetrameric $\alpha_2\beta_2$ system using sucrose gradient centrifugation in comparison to conventional quench experiments in high-pressure cells.

MATERIALS AND METHODS

Tryptophan synthase β_2-dimers were purified from the A2/F'A2 mutant strain of *E. coli* and stored frozen at −75 °C according to Bartholmes et al. (1976). Apo-β_2-subunit was prepared by incubating the holoenzyme with 3 mM hydroxylammonium chloride followed by extensive dialysis (DeMoss, 1962; Bartholmes et al., 1980). The α-subunit was prepared from *E. coli* strain B_8 and stored as ammonium sulfate precipitate at 4 °C as described by Kirschner et al. (1975). Enzyme concentrations were determined according to Bradford (1976). Enzymatic activity of the β_2-subunit was measured in the presence of a 5-fold excess of α-subunit as described previously (Miles & Moriguchi, 1977). The conversion of indoleglycerol phosphate to indole and glyceraldehyde phosphate in a coupled system with glyceraldehyde-3-phoshpate dehydrogenase was used to measure the activity of the α-subunit in the presence of an excess of β_2-subunit (Creighton, 1970).

Chemicals. Pyridoxal phosphate (PLP)[1] and dithioerythritol (DTE) were purchased from Serva (Heidelberg, Germany). Indoleglycerol phosphate was synthesized from fructose 1,6-diphosphate and indole in a hollow-fiber membrane reactor using aldolase, triose-phosphate isomerase, and tryptophan synthase (Schwarz et al., 1992). All other chemicals were of A-grade purity from Merck (Darmstadt). Millipore Q purified water was used throughout.

Buffer. Unless stated otherwise 0.1 M triethanolamine hydrochloride (pH 7.8 at 10 °C), 0.1 M NaCl, 2.0 mM DTE, and 0.5 mM EDTA were used as the standard buffer. Since deprotonation of the buffer cation does not create additional charges, the pressure effect on the pH is expected to be negligible (Neumann et al., 1973).

High-Pressure Experiments. Determination of pressure-dependent deactivation and dissociation of the $\alpha_2\beta_2$ complex were performed in an autoclave with a thermostated high-pressure cell that has been described elsewhere (Fleischmann et al., 1974). Enzyme samples (0.1 mg/mL β_2, 0.3 mg/mL α) were incubated at 10 °C for defined time intervals. After pressure release, the protein solution was immediately removed, and the enzymatic activity of the β_2-reaction was measured under normal pressure at 25 °C. To prevent reactivation during the test, the experimental conditions for determining the kinetics of dissociation and association, respectively, had to be varied slightly by adding 10 μg of trypsin/mL of test medium, and the temperature had to be lowered from 37 to 25 °C (Seifert et al., 1982).

Absorbance, under high pressure, was measured on a Zeiss DMR 10 spectrophotometer with a quarz pillbox cell (LeNoble & Schlott, 1976) using a special filling technique (Wieland & van Eldik, 1989).

Sedimentation velocity experiments, using a density gradient, were performed in a Beckmann L7-65 ultracentrifuge equipped with an SW 41 Ti swing-out rotor (radial distances r_0 and r_{max} of the meniscus and the bottom: 6.74 and 15.31 cm, respectively). Applying 500 μL of enzyme solution (1 mg/mL β_2, 3 mg/mL α) on top of a 5−20% sucrose gradient (in 0.1 M TEA, pH 7.8, at 10 °C, 0.1 M NaCl, 2.0 mM DTE, and 0.5 mM EDTA) centrifugation time at 40000 rpm and 10 °C was varied. After centrifugation, samples were fractionated to determine the position of the bands by activity measurements and SDS gel electrophoresis. The latter was performed in the presence of sodium dodecyl sulfate according to Laemmli (1970). Gels contained 15% acrylamide plus *N,N'*-methylenebisacrylamide and had 1% cross-linker ammonium persulfate. Gels were stained with Coomassie blue or silver nitrate.

Proteolysis of High-Pressure Samples by Trypsin. Bovine pancreatic trypsin (10 200 IU/mg) was made up at 0.1 mg/mL in 1 mM HCl. After high-pressure incubation for different times, proteolysis was performed at 25 °C in the enzymatic test mixture [1 mL of Test-Mix, 100 μL of protein solution and 100 μL of trypsin (0.1 mg/mL)] and stopped by the addition of 10 μL of trypsin inhibitor from soybean (1 mg/mL).

Reactivation Kinetics. Enzyme samples were reconstituted under normal pressure at 10 °C after complte dissociation of the $\alpha_2\beta_2$ complex under high pressure. The reaction kinetics were measured by taking aliquots at defined times and detecting the enzymatic activity in the β_2-reaction.

RESULTS

High-Pressure Stability of Isolated α-Subunit. Different methods were applied to examine the effect of high hydrostatic pressure on the α-subunit of tryptophan synthase from *E. coli.* Enzymatic activity is the most sensitive indicator for assessing the native structure of the enzyme. After high-pressure incubation at 10 °C, pH 7.8, and 2 kbar for 2 h, the enzyme was tested under different conditions. To prevent possible refolding reactions during the test, varying PEG concentrations were added to the test medium. Even after long incubation periods, no significant decrease in activity could be determined. Another method to exclude renaturation is the addition of trypsin to the test mixture to selectively proteolyze denatured α-chains (Seifert et al., 1982; Chan et al., 1973). This experimental setup also showed no differences in activity with respect to the native enzyme tested under the same conditions.

Since equilibrium between structural conformations of the protein can be influenced by temperature and pH changes as well as by high pressure, these parameters were varied. Solutions of α-subunit were incubated at 2 kbar in a temperature range from 5 to 37 °C and a pH range from 5.5 to 7.8. Again, catalytic activity remained constant.

[1] Abbreviations: EDTA, (ethylenedinitrilo)tetraacetic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PLP, pyridoxal 5'-phosphate; DTE, dithioerythritol; PEG, poly(ethylene glycol); TEA, triethylamine.

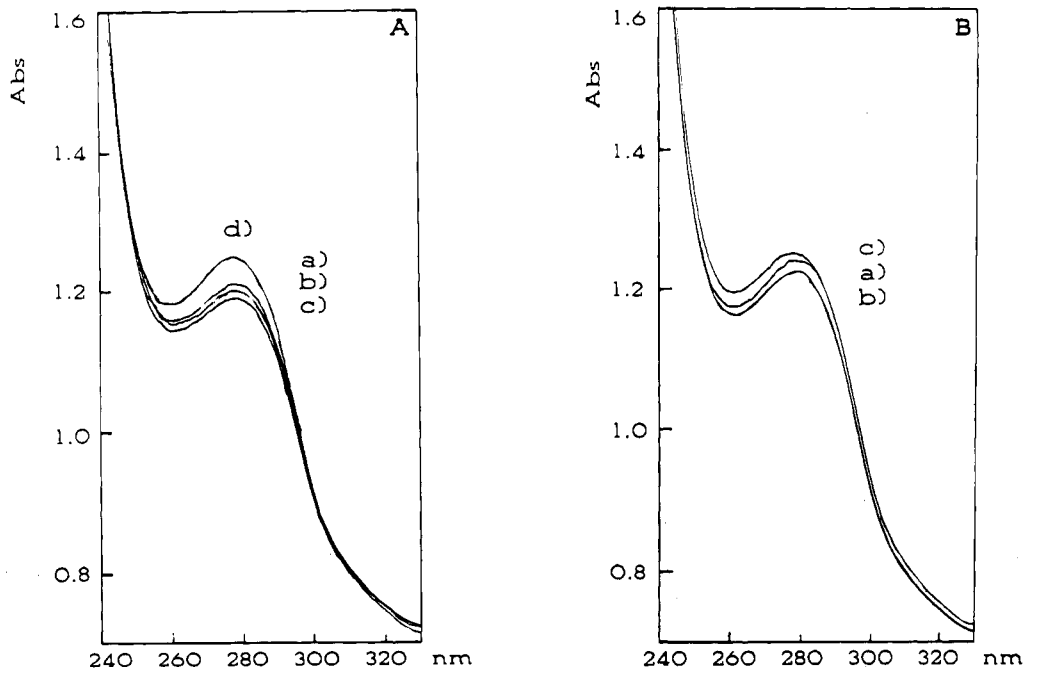

FIGURE 1: Absorption spectra of α-subunit from tryptophan synthase 0.1 mg/mL in 0.1 M Tris, pH 7.8, containing 0.1 mM DTE and 0.5 mM EDTA. (A) Spectra of the enzyme (a) without pressure and measured under high pressure: (b) 30-min, (c) 2-h, (d) 4-h incubation at 1500 bar, 25 °C. (B) Spectra of α-subunit (a) at 24 h at 1500 bar, 25 °C, and of the same sample (b) 1 min and (c) 3 h after pressure release.

In addition, spectroscopic properties like UV absorption and fluorescence emission of the protein before, during, and after high pressure were compared and found to be quite similar. Figure 1A shows UV absorption of a buffered solution of tryptophan synthase α-subunit at atmospheric and at high pressure (1.5 kbar, different incubation times). Figure 1B illustrates absorption spectra at different times after immediate release of pressure. Only minimal irreversible alterations of intensity are observed, as shown by the spectra after pressure incubation. Taken together, these results demonstrate that the α-subunit remains in its native state when exposed to 2 kbar.

Pressure-Induced Dissociation of the β_2-Component of the Bienzyme Complex. (A) General Considerations. Deactivation of the β_2-dimer, within the tetrameric $\alpha_2\beta_2$ bienzyme complex at high pressure, is a result of complex structural changes finally leading to dissociation into isolated β-chains (Seifert, 1983). Therefore $\alpha_2$apoβ_2 as well as $\alpha_2$holoβ_2 are incubated at high pressure in standard buffer for defined time intervals and tested for activity in reaction 3 after pressure release. A defined value of residual activity is achieved for each pressure.

Under the approximate assumption of a dissociation process

$$\beta_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} 2\beta \tag{4}$$

data analysis leads us to the actual β_2 concentration, calculated from enzymic activity, at any given time

$$\beta_2 = \frac{[\beta_2^0]\{[\beta_2^0] + [\beta_2^\infty]\exp(k_1 tQ)\}}{[\beta_2^0]\exp(k_1 tQ) + [\beta_2^\infty]} \tag{5}$$

$$Q = \frac{[\beta_2^0] + [\beta_2^\infty]}{[\beta_2^0] - [\beta_2^\infty]} \tag{6}$$

where $[\beta_2^0]$ is the concentration of the β_2-dimers at zero time and $[\beta_2^\infty]$ is the concentration at equilibrium (Seifert, 1983).

Linearization of the deactivation kinetics according to

$$-k_1 t = \frac{[\beta_2^0] - [\beta_2^\infty]}{[\beta_2^0] + [\beta_2^\infty]} \ln \frac{([\beta_2] - [\beta_2^\infty])[\beta_2^0]}{[\beta_2^0]^2 - [\beta_2^\infty][\beta_2]} \tag{7}$$

yields the rate constant of dissociation k_1, which is connected with the rate constant of association k_{-1} according to

$$k_{-1} = k_1 \frac{[\beta_2^\infty]}{4([\beta_2^0] - [\beta_2^\infty])^2} \tag{8}$$

The volume of activation may be obtained by a plot of ln k_1 and ln k_{-1} vs pressure according to

$$\ln k_{1,-1(p,T)} = \ln k_{1,-1(p_0,T)} - \frac{\Delta V_{1,-1}^{\ddagger}}{RT} p \tag{9}$$

To determine the midpoint of the $\beta_2 \rightleftarrows 2\beta$ transition ($p_{1/2}$), the final values after reaching equilibrium are plotted vs pressure. The solid transition curves can be calculated according to

$$\ln K_{p,T} = \ln K_{p0,T} - \frac{\Delta V}{RT} p \tag{10}$$

Pressure-dependent dissociation of oligomeric $\alpha_2\beta_2$ enzyme can be independently detected using the sedimentation velocity of the protein layered on top of a preformed sucrose gradient. The pressure gradient along the tube depends on

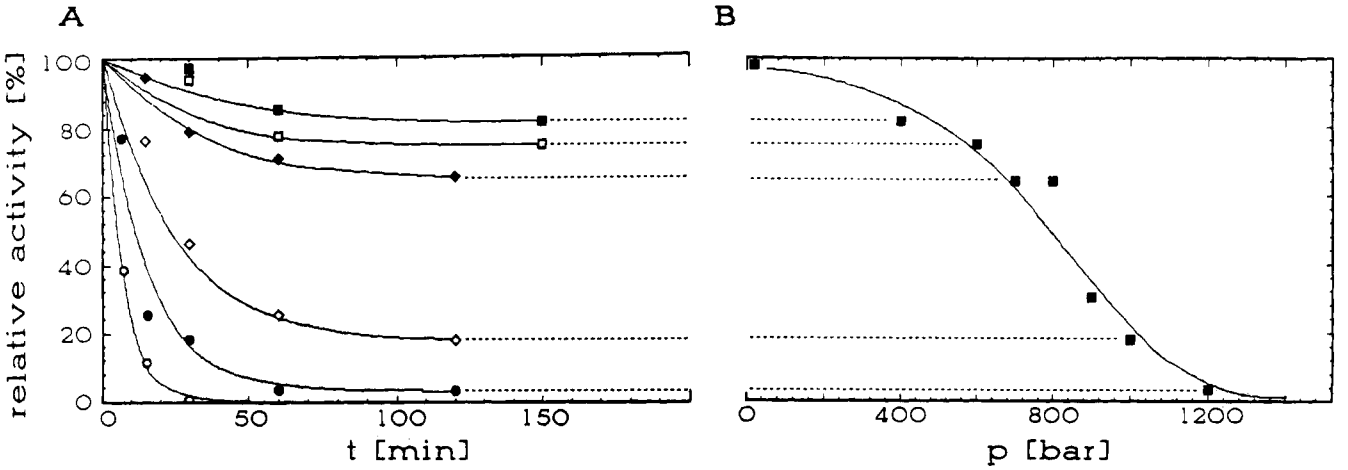

FIGURE 2: (A) Dissociation kinetics of the central apo-β_2 part of $\alpha_2$apoβ_2 (in the presence of 5-fold excess of α-subunit) for selected pressures at 10 °C. Determination of the residual activity after defined incubation times at (■) 400, (□) 600, (♦) 800, (◇) 1000, (●) 1200, and (○) 1400 bar. Solid lines were calculated from linearization of the deactivation kinetics according to eq 5 with β_2^∞ and k_1 from Tables 1 and 2. (B) Dissociation equilibrium profile including the end points from panel A of the apo-β_2-dimer of tryptophan synthase (in the presence of a 5-fold excess of α subunit). Solid line was calculated according to eq 10 with K (1 bar) = 4.2×10^{-9} M as equilibrium constant and $\Delta V = -196$ mL/mol as reaction volume.

the given dimensions according to

$$p(r) = p(r_0) + \frac{1}{2}\varrho\omega^2(r^2 - r_0^2) \qquad (11)$$

where p is the pressure, r is the distance from the center of rotation, ϱ is the density of solution, and ω is the angular velocity. Ignoring the density gradient, maximum speed (40 000 rpm) yields a pressure range from $p(r_0) = 1$ bar at the meniscus up to $p(r_{max}) = 1658$ bar at the bottom.

(B) Pressure-Induced Inactivation and Dissociation of the apo-β_2-Component. The solid lines in Figure 2A are calculated on the basis of a dissociation process. They optimally fit the measured kinetics of inactivation of the apoenzyme with an excess of α-subunit under different pressures. With increasing pressure, the enzyme is more rapidly inactivated. The activation volume for the forward reaction is $\Delta V_1^\ddagger = -89$ mL/mol and for the backward reaction is $\Delta V_{-1}^\ddagger = 107$ mL/mol. Figure 2B shows the pressure-dependent inactivation of the apo-β_2-dimer composed of the equilibrium values from Figure 2A and some additional equilibrium data. The solid curve, the equilibrium constant at atmospheric pressure K(1 bar) = 4.19×10^{-9} mol/L, and the reaction volume $\Delta V = -196$ mL/mol are obtained from the linearization according to eq 10.

When the same mixture of $\alpha_2$apoβ_2 with an excess of α-subunit is sedimented in a sucrose gradient, two independent peaks result: one exhibiting only α-activity and a second one showing exclusively β_2-activity (Figure 6). In addition, by assaying the system via reaction 3, a significant decrease in sedimentation velocity of the β_2-dimer can be observed at about 700 bar. Figure 5 shows the time dependence of sedimentation and the corresponding pressures calculated according to eq 11. Lines are obtained according to Martin and Ames (1961)

$$\frac{r_1}{r_2} = \frac{v_1}{v_2} = \left(\frac{M_1}{M_2}\right)^{2/3} \qquad (12)$$

with $\alpha_2\beta_2$ complex ($M_r = 145\,000$), β_2-dimer ($M_r = 86\,000$), and β-monomer ($M_r = 43\,000$). These results unequivocally prove dissociation of β_2-dimers into isolated β-chains.

Table 1: Pressure Dependence of the Rate Constants of Deactivation (k_1) and Reactivation (k_{-1}) of the β_2-Dimer of Tryptophan Synthase[a]

	p (bar)	k_1 (s^{-1})	k_{-1} (L mol^{-1} s^{-1})	β_2^∞ (%)
apoenzyme	1	1.0×10^{-5} (1.0×10^{-5})	2.5×10^3 (1.1×10^4)	100 (99)
	400	5.8×10^{-5}	4.1×10^2	81 (88)
	600	1.1×10^{-4}	1.6×10^2	75 (60)
	800	1.4×10^{-4}	6.5×10^1	65 (26)
	1000	5.1×10^{-4} (9.0×10^{-4})	2.6×10^1 (3.7)	18 (4)
	1200	1.1×10^{-3}	1.0×10^1	4 (0)
	1400	2.5×10^{-3}	4.1	0
holoenzyme	1	3.5×10^{-9} (1.7×10^{-8})	3.8×10^9 (1.7×10^8)	100 (100)
	1000	1.4×10^{-5} (8.5×10^{-5})	2.5×10^1 (2.3×10^{-1})	60 (3)
	1200	1.1×10^{-4}	0.6	0
	1400	4.0×10^{-4}	1.3×10^{-2}	0

[a] In the presence of a 5-fold excess of the corresponding α-subunit and residual equilibrium concentration (β_2^∞). Values in parentheses refer to measurements without α-subunit of the isolated β_2-dimer (Seifert et al., 1985).

(C) Pressure-Induced Inactivation and Dissociation of the holo-β_2-Component. Figure 3A shows the time-dependent β-deactivation of the holo enzyme complex with an excess of α-subunit at different pressures. The kinetics of inactivation of the holo-β_2-enzyme in the presence of α-subunit are slowed down distinctly in comparison to the apoprotein and to the holo-β_2-enzyme in the absence of α-subunit (Seifert, 1985). Again, the individual rate constants k_1 and k_{-1} (Table 1) are obtained by linearization of the deactivation kinetics (Figure 4, parts A and C). The corresponding activation volumes are $\Delta V_1^\ddagger = -195$ mL/mol and $\Delta V_{-1}^\ddagger = 437$ mL/mol.

The deactivation profile reveals significant differences to the apoprotein. The cooperativity of the process in the presence of pyridoxal phosphate is appreciably higher in accordance with the large reaction volume $\Delta V = -632$ mL/mol. An equilibrium constant at normal pressure K(1 bar) = 9.76×10^{-19} mol/L (Table 2) was used to fit the data. The corresponding equilibrium transition curve is shown in Figure 3B. The midpoint of the transition $p_{1/2}$ is shifted

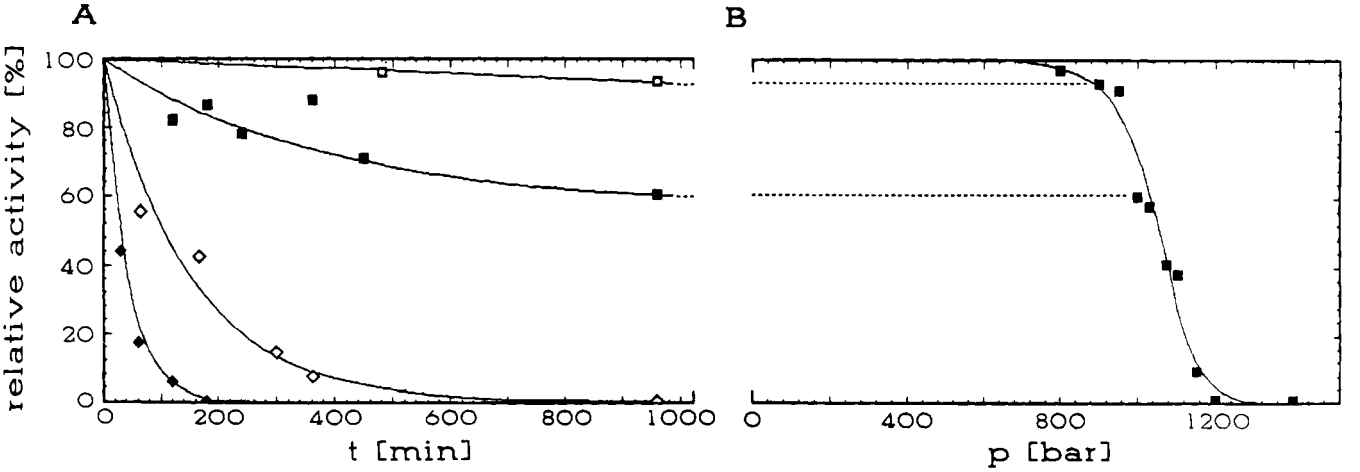

FIGURE 3: (A) Dissociation kinetics of holo-β_2 (in presence of a 5-fold excess of α-subunit) upon incubation at selected pressures at 10 °C determined from the residual activity at defined times: (□) 900, (■) 1000, (◇) 1200, and (◆) 1400 bar. Solid lines were calculated from linearization of the deactivation kinetics according to eq 5 with β_2 and k_1 from Tables 1 and 2. (B) Equilibrium transition profile of the holo-β_2-dimer of tryptophan synthase (in the presence of a 5-fold excess of α-subunit), final values from panel A plus additional values measured at different pressures. Solid line was calculated according to eq 10 with K (1 bar) = 9.8×10^{-19} M as equilibrium constant and $\Delta V = -632$ mL/mol as reaction volume.

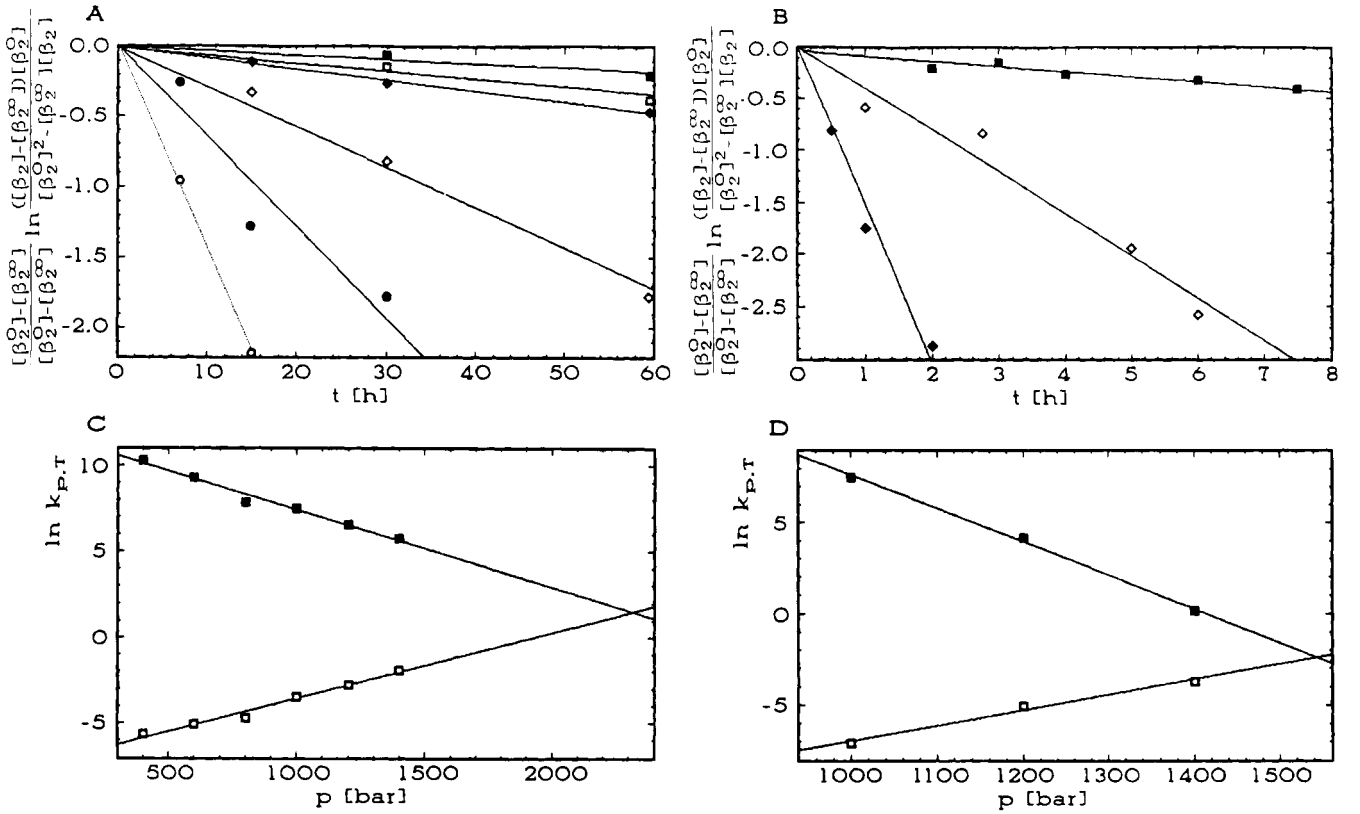

FIGURE 4: (A) Linearization of the deactivation kinetics of the apoenzyme according to eq 7 with β_2^∞ from Tables 1 and 2 and $\beta_2^0 = 100\%$. Symbols are the same as in Figure 2. (B) Semilogarithmic plot of the rate constants of dissociation (k_1) and association (k_{-1}) calculated from eq 9 (Tables 1 and 2) vs pressure yielding the activation volumes for the forward and backward reaction: (■) in k_1, (□) in k_{-1}. (C) Linearization of the deactivation kinetics of the holoenzyme. Symbols are the same as in Figure 3. (D) Semilogarithmic plot of the rate constants of dissociation and association vs pressure yielding the activation volumes for the forward and backward reaction: (■) in k_1, (□) in k_{-1}.

significantly to higher pressures: 1057 bar as compared to 749 bar for the apoenzyme.

When $\alpha_2$holoβ_2 was sedimented in the presence of an excess of α-subunit, a more rapidly sedimenting peak appeared containing α- and β_2-subunits (Figures 5 and 6). The excess of α-protein was found at the position calculated for the free α-subunit. Cases where α and β_2 appear on positions with s-values between those for tryptophan synthase $\alpha_2$holoβ_2 complex and α-subunit could be considered as partial association products with $\alpha\beta_2$ stoichiometry. Even at 1600 bar the holoenzyme complex is only incompletely dissociated (Figure 6). Therefore, additional high-pressure experiments in an autoclave were performed in the presence of 13% sucrose.

The stabilizing effect of sucrose, in general, holds for pressure-induced denaturation, dissociation, and inactivation

Table 2: Activation Volumes $\Delta V_{1,-1}^{\ddagger}$ for the Forward and Backward Reactions As Calculated from Rate Constants According to Eq 9[a]

	$\Delta V_1^{\ddagger}$ (mL/mol)	$\Delta V_{-1}^{\ddagger}$ (mL/mol)	K (1 bar) (mol/L)	ΔV (mL/mol)	$p_{1/2}$ (bar)
apoenzyme	−89 (−105)	107 (185)	4.19×10^{-9} (4.5×10^{-10})	−196 (−290)	749 (690)
holoenzyme	−195 (−190)	437 (480)	9.76×10^{-19} (1.2×10^{-16})	−632 (−670)	1057 (870)

[a] Equilibrium constants at atmospheric pressure K (1 bar), reaction volume ΔV (eq 10), and the midpoint of the equilibrium transition ($p_{1/2}$). Values in parentheses refer to measurements without α-subunit of the isolated β_2-dimer (Seifert et al., 1985).

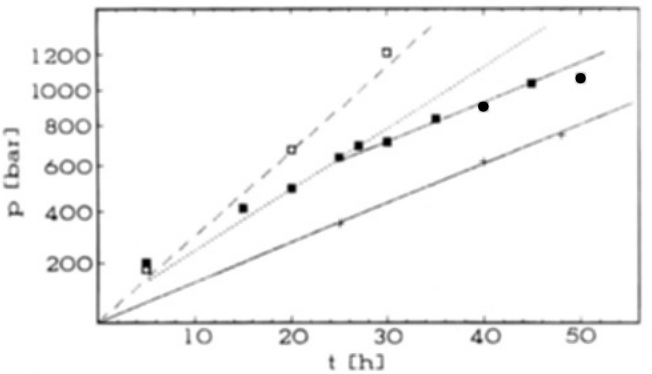

FIGURE 5: Sucrose gradient centrifugation of the apo and holo complex of tryptophan synthase. Sedimentation in 5−20% sucrose gradient in standard buffer at 40000 rpm, 10 °C. After varying sedimentation times, the distances of the proteins from the center of rotation were determined by enzyme activity of the bands in the tube. The corresponding pressures are calculated according to eq 11. (+) Sedimentation of ovalbumin (M_r = 45 000) measured as monomeric reference, (■) $\alpha_2$apoβ_2 (1 mg/mL β_2, 3 mg/mL α), and (□) $\alpha_2$holoβ_2 (same concentrations plus 40 μM PLP). (− −) Sedimentation velocities of $\alpha_2\beta_2$ complex, (M_r = 145 000), (···) β_2-subunit (M_r = 86 000), and (−) β-monomer (M_r = 43 000) calculated according to eq 12.

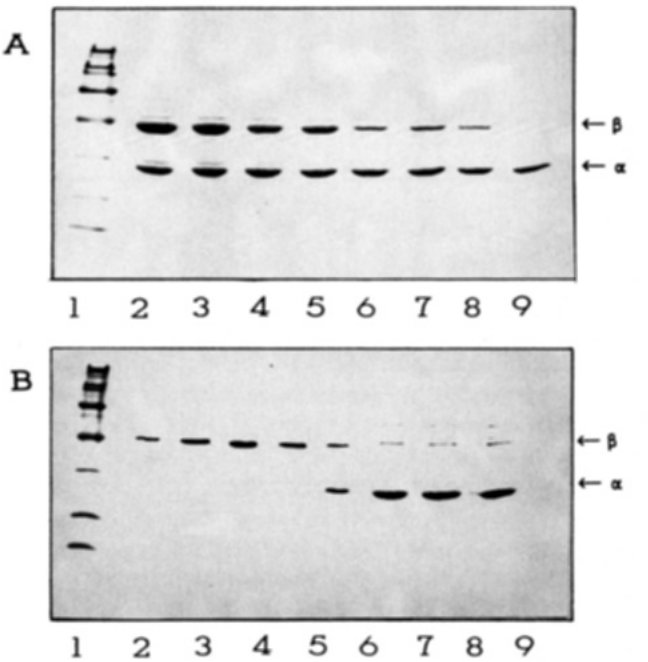

FIGURE 6: SDS−polyacrylamide gel electrophorese. (A) Protein fractions (0.5 mL) after 50-h sedimentation at 500 μL of $\alpha_2$holoβ_2 in standard buffer (1 mg/mL β_2, 3 mg/mL α) layer on top of a 5−20% sucrose gradient containing 40 μM PLP. Lane 1, molecular mass markers: myosin (M_r = 200 000), β-galactosidase (M_r = 116 250), phosphorylase *b* (M_r = 97 400), bovine serum albumine (M_r = 66 200), ovalbumin (M_r = 45 000), carbonic anhydrase (M_r = 31 000), soybean trypsin inhibitor (M_r = 21 500) and lysozyme (M_r = 14 400). Lane 2, fraction (0.5 mL) from the bottom of the centrifugation tube, radial distance 15.31 cm from the center of rotation with $p(r_{max})$ = 1658 bar; lanes 3−9, following fractions, each 0.5 mL. (B) Protein fractions (0.5 mL) after 50-h sedimentation of $\alpha_2$apoβ_2 (1 mg/mL β_2, 3 mg/mL α) in 5−20% sucrose, same lanes as in panel A.

(Lee et al., 1979, Lee & Timasheff, 1981, Arakawa & Timascheff, 1982; Timascheff et al., 1982; Seifert et al., 1984). Quench experiments, in the presence of 13% sucrose, show a significant shift of the equilibrium transition toward higher pressures (data not shown). The midpoint of the transition ($p_{1/2}$) of the $\alpha_2$holoβ_2 tryptophan synthase complex is $p_{1/2}$ = 1057 bar in the absence of sucrose and $p_{1/2}$ = 1385 bar in buffer solution with 13% sucrose. Furthermore, the calculated dissociation curve exhibits decreased cooperativity and a lower value of the reaction volume of the pressure-induced denaturation (ΔV = −632 mL/mol without sucrose, ΔV = −395 mL/mol with 13% sucrose). Complete dissociation is achieved at 1750 bar, a value which cannot be reached with sucrose gradient centrifugation in the swing-out rotor used in this investigation.

In an attempt to show that complete dissociation of the $\alpha_2$holoβ_2 into single peptide chains takes place, limited trypsin proteolysis was performed with $\alpha_2$holoβ_2 complex incubated at high pressure in comparison to the untreated enzyme (Figure 7). Applying SDS−PAGE, the latter is expected to be proteolysed into α-1 (M_r = 20 500) and α-2 fragments (M_r = 8300) and unaffected β-chains (lane 1) (Miles & Higgins, 1979). Trypsinolysis of the isolated native β_2-subunit yields β-F_{1a}, β-F_{1b}, and β-F_2 fragments as shown earlier by Högberg-Raibaud and Goldberg (1977a,b). Monomeric β is cleaved totally into small products undetectable in the gel. High-pressure samples of $\alpha_2$holoβ_2 treated with trypsin therefore exhibited different degrees of fully digested α- and β-fragments and active modified complex in dependence on incubation time (data not shown). Figure 7 (lanes 5−9) shows that after complete deactivation at 1700 bar, followed by pressure release, neither β-protein nor β-fragments can be observed after varying incubation times with trypsin. The α-subunit is rapidly (5 min) cleaved at Arg 188, yielding an instable α′ derivative that undergoes complete degradation within 60 min (Miles & Higgins, 1978). After reconstitution of the $\alpha_2$holoβ_2 complex, the corresponding SDS bands (lane 3) are indistinguishable from those of the native complex (lane 2).

Reactivation Kinetics of apo- and holo-β_2-Subunits in the Presence of α-Subunit. The kinetics of renaturation of the enzyme can be followed by the recovery of catalytic activity of the β_2-subunit. Starting material is the completely deactivated protein (25 min, 2 kbar, 10 °C). After pressure release, the observed progress curve in the absence of the cofactor (Figure 8A) is characterized by a half-time of 18 min. The process of reactivation reaches an equilibrium value of about 92%. As demonstrated in Figure 8A, there is no significant difference in the time course of reactivation of completely dissociated $\alpha_2\beta_2$ complex and of pressure-dissociated β_2-protein reactivation in the presence of native α-subunit added at the beginning of the reconstitution process.

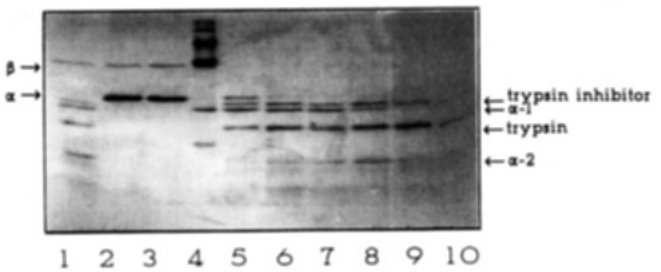

FIGURE 7: SDS−polyacrylamide gel electrophorese of tryptophan synthase $\alpha_2\beta_2$ complex with a 5-fold excess α-subunit. Lane 1, native $\alpha_2\beta_2$ complex incubated for 10 min with trypsin at 25 °C; then the reaction was stopped with trypsin inhibitor. Lane 2, native sample without any treatment. Lane 3, sample renaturated after 3 h, 1700 bar, 10 °C. Lane 4, molecular mass markers: myosin (M_r = 200 000), β-galactosidase (M_r = 116 250), phosphorylase *b* (M_r = 97 400), bovine serum albumine (M_r = 66 200), ovalbumin (M_r = 45 000), soybean trypsin inhibitor (M_r = 21 500), and lysozyme (M_r = 14 000). Lane 5, sample after 3 h, 1700 bar, 10 °C incubated with trypsin for 1 min: upper band, native α-subunit; second band, trypsin inhibitor; third one, α-1 fragment; fourth band, trypsin; lowest band, α-2 fragment. Lanes 6−9, incubation with trapsin for 5, 10, 20, and 60 min. Lane 10, bovine pancreatic trypsin and trypsin inhibitor from soybean used in the experiments of lanes 1 and 5−9.

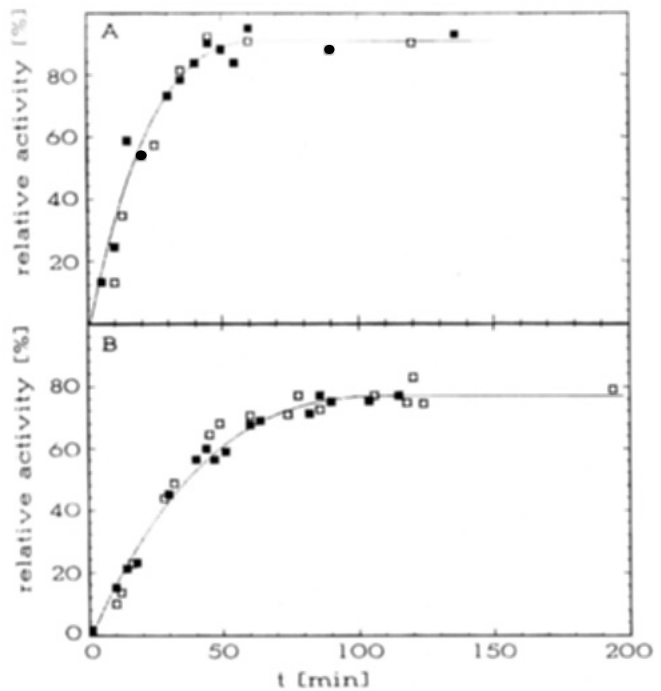

FIGURE 8: (A) Kinetics of renaturation of the apo-β_2-subunit of tryptophan synthase. (□) Dissociation of $\alpha_2$apoβ_2 (0.1 mg of apo-β_2- and 0.3 mg of α-subunit/mL) in standard buffer for 25 min at 2.0 kbar (10 °C) followed by reassociation (standard buffer, 10 °C) measured via enzymatic activity in reaction 3 (25 °C). (■) Dissociation of apoβ_2 (0.1 mg of apo-β_2/mL) in standard buffer under the same conditions followed by reassociation in standard buffer containing native α (0.3 mg of α-subunit/mL). (B) Kinetics of renaturation of the holo-β_2-subunit of tryptophan synthase. (□) Dissociation of $\alpha_2$holoβ_2 (0.1 mg of holo-β_2- and 0.3 mg of α-subunit/mL) in standard buffer containing 40 μM PLP for 25 min at 2.0 kbar (10 °C) followed by reassociation (same buffer, 10 °C) measured via enzymatic activity (25 °C). (■) Dissociation of holoβ_2 (0.1 mg of holo-β_2/mL) under the same conditions followed by reassociation in standard buffer containing PLP and α (0.3 mg of α-subunit/mL). Solid lines represent curves fit to the data.

The yield of reactivation of the holoenzyme is significantly lower, and the process is slower. At equilibrium only 78% activity is regained, with a half-time of 27 min. The kinetics of reactivation of holo β_2 were determined in the presence of pressure incubated as well as native α-subunits (Figure 8B). In accordance with the results in the absence of the cofactor, the curves with pressurized and unpressurized α respectively are similar.

DISCUSSION

As shown in recent publications (Seifert et al., 1982, 1984a,b, 1985, Silva et al., 1986), the isolated β_2-subunit of tryptophan synthase is an extremely stable dimer that exhibits reversible high-pressure deactivation paralleled by dissociation into monomers. The interaction of the α- and β_2-subunits in the bienzyme complex represents an example for the control of both the structure and the catalytic efficiency of an enzyme due to mutual stabilization of its constituent subunits (Yanofsky & Crawford, 1972). For all that, we are inclined to suppose that high-pressure inactivation of the native αββα bienzyme complex for reaction 3 is also due to dissociation of the β_2-component into β-monomers. To prove this hypothesis, the influence of the α-subunit on the structural stability of the β_2-subunit under high pressure has been investigated.

Since such an approach requires a sufficiently stable α-subunit, we first measured enzyme activity, UV absorption, and fluorescence spectra of the α-chains under high pressure. As expected from the literature (Heremans, 1982, Weber & Drickamer, 1983) for monomeric proteins, no significant pressure effects on the isolated α-subunit were detectable.

With the described experimental approach, combining activity measurements with sedimentation experiments, we could unequivocally demonstrate that deactivation of the apo-β_2-component of the $\alpha_2\beta_2$ complex under high pressure is accompanied by complete dissociation into enzymatically inactive β-monomers. On the other hand, regarding the isolated β_2-dimer (Seifert et al., 1982, 1983), it has been quantitatively analyzed that the kinetics not only of dissociation but also of reassociation as measured via hybridization between native and chemically modified β-chains parallel the respective alteration of enzymatic activity. On the basis of these earlier observations, we calculated the degree of pressure-dependent dissociation of the β_2-subunit in the presence of the corresponding α-subunits via determination of the residual β_2-activity as a measure of the concentration of native dimers. Additionally, the SDS−PAGE results of trypsinolysis of the pressure-denatured $\alpha_2\beta_2$-protein indicate that, directly after pressure release, a mixture of denatured β-forms and native α-subunits is present (Figure 7). Obviously, renaturation to yield a trypsin-resistant β-species must be slow compared to the time lapse between pressure release and determination of residual activity. Thus, pressure deactivation and reconstitution experiments of the β_2-subunit at low temperature (10 °C) and with trypsin present in the enzymatic test are a suitable method to obtain reliable kinetic data.

For the dissociation of holo- and apo-β_2 dimers in the presence of α-subunits, large negative reaction and activation volumes were found. Considering Braun−Le Chatelier's principle, it is obvious that a negative activation volume $\Delta V_1^{\ddagger}$ leads to an increase in reaction rate with increasing pressure. A negative reaction volume ΔV causes a shift of the equilibrium toward the products. Comparing the results of our experiments with the $\alpha_2\beta_2$ complex to those of the

isolated β_2-dimer (Seifert et al., 1985), our findings underline the extremely high stability of the β_2-component in the tryptophan synthase complex, especially in the presence of its cofactor pyridoxal 5′-phosphate that is bound to K87 in the active center of the β-chain (Creighton & Yanofsky, 1966, Wilson & Crawford, 1965).

Consequently, the midpoint of the equilibrium transition curve for the holoenzyme (Table 1) is considerably shifted to higher pressures (870 bar for β_2 and 1057 bar for $\alpha_2\beta_2$, respectively) because of the tight interaction between α- and β_2-subunits. This result is corroborated by the finding that the transition curve exhibits increased cooperativity. Equilibrium dissociation constants (Table 2) for the $\alpha_2$holoβ_2 complex are found to be smaller by a factor of 120 as compared to the isolated holo-β_2-subunit (Seifert, 1985).

In the case of the apoenzyme, the stabilizing effect of the α-subunit is much less pronounced. The corresponding dissociation constants only differ by a factor of 10 (Table 2), and the midpoints of the transition curves are located at 680 bar for β_2 and at 790 bar for $\alpha_2\beta_2$, respectively. This finding is not unexpected. We can deduce from Figure 5 that, in the absence of the cofactor, the α-subunit dissociates from β_2 at comparably low pressure.

The reconstitution of the oligomeric protein after pressure release is an essentially reversible process including both specific homologous and heterologous interactions of the α- and β-subunits. Since the α-enzyme is known to stabilize the native structure of the bienzyme complex (Miles, 1979, 1991), it may also influence the kinetics of reconstitution by accelerating rate-limiting steps or by stabilizing folding intermediates and/or the final product of reconstitution.

As indicated by the high yield of reactivation (92%) of the apo complex after pressure release, only minimal interference between reconstitution and unproductive aggregation of the enzyme appears to occur. Regarding its enzymological properties, the enzyme renaturing with $t_{1/2}$ = 18 min is indistinguishable from the native one.

Comparing these results with the reactivation of apo-β_2 in the absence of the corresponding α-chains (Seifert, 1983), the α-subunit has no significant effect on the rate of association of apo-β_2. Therefore, we conclude that the previously observed cooperative binding of α-subunits to apoβ_2, which is characterized by K_{d1} = 29 μM and K_{d2} = 0.8 μM, respectively (Bartholmes & Teuscher, 1979), only occurs after the independent refolding and association of the two β-monomers to yield a native β_2-subunit is complete. This view is corroborated by the close similarity of the physical parameters deduced from equilibrium experiments (as compiled in Tables 1 and 2).

The reconstitution process of holo-β_2 in the presence of the α-subunit is significantly less effective and slower when compared to the isolated holo-β_2-subunit [$\alpha_2$holoβ_2, $t_{1/2}$ = 27 min, 78% activity regain, holoβ_2, $t_{1/2}$ = 17 min, 96% activity regain (Seifert et al., 1982)]. The mechanism of reconstitution of the holo complex is characterized by early interactions of native α-subunits with β-chains still involved in regaining the correct physiological tertiary and quaternary structures. Additional evidence supporting this view may be derived from the extremely tight anticooperative binding of α-subunits to holoβ_2 with K_d = 0.26 nM for the first association step [$\alpha\beta_2$ (Lane et al., 1984)]. It remains an open mechanistic question whether the intermediate reacting untimely with α-subunits is an early stage of a folding monomer or a still uncorrectly folded dimer in the process of final reshuffling. On the one hand, the above-mentioned hybridization approach (Seifert et al., 1982), which is potentially capable of probing the presence of monomers in a renaturation mixture, points to a late dimerization step. On the other hand, Silva et al. (1986) describe an HPLC experiment with renaturing β-chains in which the previously pressure-dissociated protein after some 2 min elutes at the same volume as the untreated β_2-dimer. Although the kinetics of reconstitution of isolated apo- and holo-β_2 are apparently quite similar, the underlying folding pathway must be different at least, in so far as disturbing interactions between α and "immature" β_2 do not occur in the absence of PLP.

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REFERENCES

Arakawa, T., & Timasheff, S. N. (1982) *Biochemistry 21*, 6536−6544.
Bartholmes, P., & Teuscher, B. (1979) *Eur. J. Biochem. 95*, 323−326.
Bartholmes, P., Kirschner, K., & Geschwind, H.-P. (1976) *Biochemistry 15*, 4712−4717.
Bartholmes, P., Balk, H., & Kirschner, K. (1980) *Biochemistry 19*, 4257−4533.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248.
Chan, W.-C., Schutt, H., & Brand, K. (1973) *Eur. J. Biochem. 40*, 533−541.
Creighton, T. E. (1970) *Eur. J. Biochem. 13*, 1−10.
Creighton, T. E. & Yanofsky, C. (1966) *J. Biol. Chem. 241*, 980−990.
DeMoss, J. A. (1962) *Biochim. Biophys. Acta 62*, 279−293.
Dicamelli, R. F., Balbinder, E., & Lebowitz, J. C. (1973) *Arch. Biochem. Biophys. 155*, 315−324.
Fleischmann, F. K., Conze, E. G., Stranks, D. E., & Kelm, H. (1974) *Rev. Sci. Instrum. 45*, 1427.
Groha, C., Bartholmes, P., & Jaenicke, R. (1978) *Eur. J. Biochem. 92*, 437−441.
Hathaway, G. M., & Crawford, I. P. (1970) *Biochemistry 9*, 1801−1808.
Henning, U., Helinsky, D. R., Chao, F. C., & Yanofsky, C. (1962) *J. Biol. Chem. 237*, 1523−1530.
Heremans, K. (1982) *Annu. Rev. Biophys. Bioeng. 11*, 1−21.
Higgins, W., Fairwell, T., & Miles, E. W. (1979) *Biochemistry 18*, 4827−4835.
Högberg-Raibaud, A., & Goldberg, M. E. (1977a) *Proc. Natl. Acad. Sci. U.S.A. 74*, 442−446.
Högberg-Raibaud, A., & Goldberg, M. E. (1977b) *Biochemistry 16*, 4014−4019.
Jaenicke, R. (1981) *Annu. Rev. Biophys. Bioeng. 10*, 1−8.
Jaenicke, R., & Koberstein, R. (1971) *FEBS Lett. 19*, 351−355.
Kirschner, K., Weischet, W. O., & Wiskocil, R. L. (1975) in *Protein-ligand interactions* (Sund, H., & Blauer, W., Eds.) pp 27−4, W. de Gruyter, Verlag, Berlin.
Klotz, I. M., Langermann, N. R., & Darnall, D. W. (1970) *Annu. Rev. Biochem. 39*, 25−62.
Laemmli, U. K. (1970) *Nature 227*, 680−685.
Lane, P., & Kirschner, K. (1984) *EMBO J. 3*, 279−287.
Lee, J. C., & Timasheff, S. N. (1981) *J. Biol. Chem. 256*, 7193−7201.
Lee, J. C., Gekko, K., & Timasheff, S. N. (1979) *Methods Enzymol. 61*, 26−49.
LeNoble, W. J., & Schlott, R. (1976) *Rev. Sci. Instrum. 47*, 770.
Martin, R. G., & Ames, N. (1961) *J. Biol. Chem. 236*, 1372−1379.
Miles, E. W. (1979) *Adv. Enzymol. Relat. Areas Mol. Biol. 49*, 127−186.

Miles, E. W. (1991) *Adv. Enzymol. Relat. Areas Mol. Biol. 64*, 93–172.
Miles, E. W., & Moriguchi, M. (1977) *J. Biol. Chem. 252*, 6594–6599.
Miles, E. W., & Higgins, W. (1978) *J. Biol. Chem. 253*, 6266–6269.
Neumann, R. C., Kauzmann, W., & Zipp, A. (1973) *J. Phys. Chem. 77*, 2687–2691.
Penniston, I. T. (1971) *Arch. Biochem. Biophys. 142*, 322–332.
Reithel, F. J. (1963) in *Advances in Protein Chemistry* (Anfinsen, C. B., Anson, M. L., & Edsall, J. T., Eds.) Vol. 18, pp 123–225, Academic Press, New York.
Schade, B. C., Rudolph, R., Lüdemann, H.-D., & Jaenicke, R. (1980a) *Biochemistry 19*, 1121.
Schade, B. C., Lüdemann, H.-D., Rudolph, R., & Jaenicke, R. (1980b) *Biophys. Chem. 11*, 257.
Schmid, G., Lüdemann, H.-D., & Jaenicke, R. (1979) *Eur. J. Biochem. 97*, 407–413.
Schwarz, T., Eberhard, G., & Bartholmes, P. (1992) *Biotechnol. Appl. Biochem 15*, 31–39.
Seifert, T. (1983) Thesis, University of Regensburg.
Seifert, T., Bartholmes, P., & Jaenicke, R. (1982) *Biophys. Chem. 15*, 1–8.
Seifert, T., Bartholmes, P., & Jaenicke, R. (1984a) *Z. Naturforsch., C: Biosci. 39C*, 1008–1011.
Seifert, T., Bartholmes, P., & Jaenicke, R. (1984b) *FEBS Lett. 173*, 381–384.
Seifert, T., Bartholmes, P., & Jaenicke, R. (1985) *Biochemistry 24*, 339–345.
Silva, J. L., Miles, E. W., & Weber, G. (1986) *Biochemistry 25*, 5780–5786.
TanEyck, L. F., & Kauzmann, W. (1967) *Proc. Natl. Acad. Sci. U.S.A. 58*, 888–894.
Tanford, C. (1968) in *Advances in Protein Chemistry* (Anfinsen, C. B., Anson, M. L., & Edsall, J. T., Eds.) Vol. 23, pp 121–282, Academic Press, New York.
Tanford, C. (1970) in *Advances in Protein Chemistry* (Anfinsen, C. B., Anson, M. L., & Edsall, J. T., Eds.) Vol. 24, pp 1–95, Academic Press, New York.
Timasheff, S. N., Arakawa, T., Inoue, H., Gekko, K., Gorbunoff, M. J., Lee, J. C., Na, G. C., Pittz, E. P., & Prakash, V. (1982) in *Biophysics of Water* (Franks, F., & Mathias, S., Eds.) pp 48–50, Wiley, Chichester, UK.
Weber, G., & Drickamer, H. G. (1983) *Q. Rev. Biophys. I 16*, 89–112.
Wieland, S., & van Eldik, R. (1989) *Rev. Sci. Instrum. 60*, 955.
Wilson, D. A., & Crawford, I. P. (1965) *J. Biol. Chem. 240*, 4801–4808.

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