Pressure Dissociation and Conformational Drift of the β Dimer of Tryptophan Synthase†

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ABSTRACT: Micromolar solutions of tryptophan synthase β_2 dimer dissociate into monomers in the pressure range of 800–1600 bars as shown by studies of the spectral shift of the intrinsic fluorescence and of the fluorescence polarization of dansyl conjugates. At 25 °C the standard change in volume on dissociation (dV^0) of the holoprotein was −162 mL mol^{-1}, and the dissociation constant at 1 bar was $K_0 = 3.7\ 10^{-10}$ M. Pyridoxal-reduced holoprotein and apoprotein had, within 10%, the same dV_0, but K_0 was decreased in the reduced protein (6×10^{-11} M) and increased in the apoprotein (3.6×10^{-9} M). At 4 °C the free energy of association of the holoprotein was reduced by 1.4 kcal mol^{-1}, but dV_0 was unchanged. In all the protein forms the decompression curves differed from the respective compression curves, indicating the loss of some free energy of association following separation of the monomers. This hysteretic behavior was largest in the apoprotein and amounted to a loss of 2.6 kcal mol^{-1} in the free energy of association. When the pressure was rapidly raised to 2.2 kbars, half-dissociation of the reduced pyridoxal β_2 dimer took approximately 12 min. Upon return to atmospheric pressure reassociation was complete in 2–3 min and half of the enzyme activity was regained in 10 min; pyridoxal fluorescence recovered more slowly with a biphasic course. The independent return of these properties and the hysteretic behavior indicate that subunit separation is followed by a conformational drift like that observed in lactate dehydrogenase dissociated by either pressure or temperature or in enolase dissociated by dilution.

The strength of association between subunits in an oligomeric protein is determined by the free energy of subunit association, but this important thermodynamic parameter has been measured in only a few out of the hundreds of well-characterized oligomeric proteins (Klotz et al., 1975). This paucity of data is attributed to the often very small dissociation constant that demands the determination of the degree of dissociation at very high dilutions. Similarly, the study of the properties of the isolated monomers in physiological solvents has proven possible in only a few cases. The dissociation of oligomeric proteins by pressure provides a method, apparently very general, of inducing their reversible dissociation at concentrations at which this process can be readily followed by several spectroscopic techniques (Paladini & Weber, 1981); King & Weber, 1986a,b). It has been repeatedly shown that pressures below 3 kbars do not affect the tertiary structure of single-chain proteins [for 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. The present paper describes the pressure-induced dissociation of β dimers of tryptophan synthase. The dissociation was followed by the decrease in the energy of the intrinsic protein fluorescence and by changes in the polarization of fluorescence of dansyl conjugates of the protein. The partial loss of subunit affinity after dissociation and changes in spectroscopic and functional properties of the associated protein after decompression were investigated. In addition to the determination of the free energy of subunit association under various conditions, our results demonstrate that the separated subunits of β_2 tryptophan synthase undergo a conformational drift (Weber, 1986). We define this "conformational drift" as the progressive loss of free energy of association of the subunits of an oligomer when these became separated following changes in pressure (King & Weber, 1986a), temperature (King & Weber, 1986b), or concentration (Xu & Weber, 1982).

Materials and Methods

Chemicals. Pyridoxal phosphate and Bis-tris-propane[1] were obtained from Calbiochem. All other reagents were of analytical grade. The water was distilled and filtered through a millipore water purification system to 18-Mohm resistance. The high-pressure cuvettes were silanized as described by Royer (1985), in order to prevent protein adhering to the quartz walls. Unless stated otherwise, the experiments were performed in the standard buffer: 0.05 M Bis-tris-propane, 1 mM EDTA, and 0.5 mM DTE at pH 7.5. A prepacked TSK gel 3000 SW column (30 cm × 7.5 mm i.d.) from Toyo Soda was used for the size-exclusion chromatography of the tryptophan synthase β_2 subunit.

Proteins and Enzyme Assays. The $\alpha_2\beta_2$ complex of tryptophan synthase was prepared from *Escherichia coli* strain *W3110 Δ trpR cysB Δ trp LD102 trpB$^+$ trpA$^+$/F′ colVE cysB$^+$ Δ trp LD102 trpB$^+$ trpA$^+$* as described by Higgins et al. (1979). The holo β_2 subunit, that is, the protein containing 1 mol of pyridoxal phosphate per monomer, was isolated from the $\alpha_2\beta_2$ complex by heat denaturation of the α subunit (Hogberg-Raibaud & Goldberg, 1977). Reduction of the Schiff base linking the cofactor to the protein was achieved

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[1] Abbreviations: Bis-tris-propane, 1,3-bis[[tris(hydroxymethyl)-methyl]amino]propane; 2,5-DNS, 2-amino-5-naphthalenesulfonate; DTE, dithioerythritol; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; pyridoxal-P, pyridoxal 5′-phosphate.

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by treatment with sodium tetrahydridoborate (Zetina & Goldberg, 1980). We shall refer to this species as "reduced holoprotein". Typically, 5 μL of 80 mM $NaBH_4$ in 0.05 N NaOH was added to a solution of holo β_2 at 1.8 mg/mL. The pyridoxal-P bound to the enzyme was fully reduced as judged by the disappearance of the fluorescence emission at 520 nm upon excitation at 410 nm and the increase of fluorescence emission at 390 nm upon excitation at 330 nm (Bartholmes et al., 1980). The solution was then dialyzed against the standard buffer overnight. Apo β_2 subunit was prepared by incubating the holoenzyme with 3 mM hydroxylammonium chloride for 3 h, followed by extensive dialysis (De Moss, 1962; Bartholmes et al., 1980). Protein concentrations were calculated from the specific absorption of the apo β_2 subunit (E_{278} = 5.8) and of the holo β_2 subunit (E_{278} = 6.05) (Miles & Moriguchi, 1977). The β_2 subunit molecular weight was considered to be 90000. The enzymatic activity of the β_2 subunit was measured in the presence of a 3-fold excess of α subunit as described previously (Miles & Moriguchi, 1977).

2-(Dimethylamino)naphthalene-5-sulfonyl-reduced β_2 subunit was prepared by adding a 5-fold molar excess of the sulfonyl chloride dissolved in dimethylformamide to a solution of reduced β_2 in 50 mM potassium phosphate, 1 mM EDTA, and 0.5 mM DTE at pH 8.0. The final concentration of dimethylformamide was about 1%. The reaction mixture was left for 8 h at room temperature, and the unbound dye was removed by extensive dialysis against 0.05 M Bis-tris-propane, 1 mM EDTA, and 0.5 mM DTE at pH 7.5. Apo β_2 was labeled with dansyl chloride by using the same procedure, except that the reaction time was 2 h.

Apparatus. The high-pressure bomb has been described by Paladini and Weber (1981a,b). Spectra were recorded on a microprocessor-controlled photon counting scanning spectrofluorometer (Royer, 1985). The spectra were corrected for wavelength response of the phototube and monochromator. Polarization measurements were made in the L format by using the photon counting polarization instrument described by Paladini (1980).

Analysis. Spectral shifts of the fluorescence can be quantitated by specification of the center of spectral mass ($\langle \nu_g \rangle$), a parameter defined by the equation

$$\langle \nu_g \rangle = \sum \nu_I F_I / \sum F_I \quad (1)$$

where F_I stands for the fluorescence emitted at wavenumber ν_I and the summation is carried over the range of appreciable values of F_I. The degree of dissociation (a) is related to $\langle \nu_g \rangle$ by the expression

$$a = (1 + Q(\langle \nu_g \rangle_p - \langle \nu_g \rangle_M)/(\langle \nu_g \rangle_D - \langle \nu_g \rangle_p))^{-1} \quad (2)$$

where Q is the ratio of the quantum yields of monomer and dimer, $\langle \nu_g \rangle_p$ is the center of spectral mass at pressure P, and $\langle \nu_g \rangle_M$ and $\langle \nu_g \rangle_D$ are the corresponding quantities for the monomer and dimer. The data of fluorescence anisotropy can be used to determine the degree of dissociation by a similar expression (Paladini & Weber, 1981):

$$a = (1 + Q(A_p - A_M)/(A_D - A_p))^{-1} \quad (3)$$

A_p,A_M, and A_D are respectively the anisotropy at pressure p and those ascribed to monomer and dimer.

RESULTS

Pressure-Induced Dissociation of Holo and Reduced Tryptophan Synthase β_2 Subunit. Figure 1A shows spectra of the intrinsic fluorescence emission of a buffer solution of holo tryptophan synthase β_2 subunit at atmospheric and at high pressure (2.2 kbars). The latter spectrum shows a large red

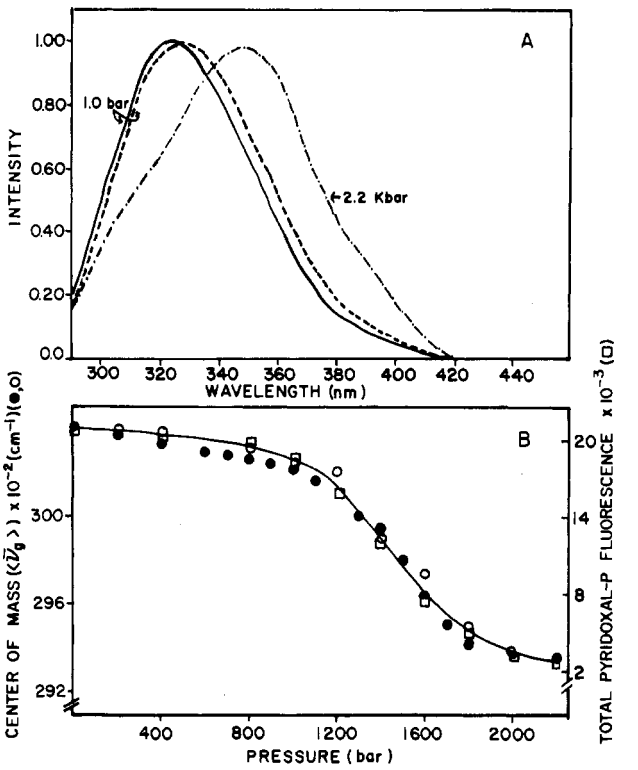

FIGURE 1: Fluorescence emission spectra of holo β_2. (A) Intrinsic fluorescence of 12 μM holo β_2, excited at 280 nm, in 0.05 M Bis-tris-propane, 1 mM EDTA, and 0.5 mM DTE at pH 7.5, measured at atmospheric pressure (—), at 2.2 kbars (-·-), and after release of pressure (---). (B) Center of spectral mass ($\langle \nu_g \rangle$) of intrinsic fluorescence of 12 μM holo β_2 vs. pressure without pyridoxal-P added (○) and in 150 μM pyridoxal-P (●). Pyridoxal-P fluorescence (excitation at 410 nm, emission at 440–640 nm) without added pyridoxal-P (□).

shift of the emission with respect to the spectrum at atmospheric pressure. Figure 1B shows a plot of $\langle \nu_g \rangle$ against pressure. The curve corresponds to a unimodal process that reaches a plateau at 2.0 kbars with a decrease of $\langle \nu_g \rangle$ of 1050 cm^{-1} between this and atmospheric pressure.

The spectrum after immediate release of pressure, also shown in Figure 1A, indicates recovery of the original value of $\langle \nu_g \rangle$ of 80–85%. The spectral transition profile was not affected by addition of excess pyridoxal-P to the medium (Figure 1B). Bound pyridoxal phosphate has a fluorescence yield nearly 20 times larger than the free form and a fluorescence polarization of 0.45. The total fluorescence intensity of pyridoxal-P bound to the protein (Figure 1B) decreased in parallel with the changes in the energy of tryptophan emission, a decrease that can be explained either by access of solvent to the bound cofactor or by its dissociation from the protein. Figure 2 shows that the shift in the center of spectral mass of tryptophan emission with pressure is dependent upon protein concentration, an absolute requirement if dissociation is to be considered the cause of the spectral shift. The apparent standard volume change upon subunit association (dV^0) was calculated in this and other instancees from the slope of the plot of $\ln (a^2/(1-a)) = \ln (K_p/4C)$ (Paladini & Weber, 1981) vs. pressure, fitting the values for degrees of dissociation between 10 and 90% to a straight line (inset of Figure 2). Here a stands for the degree of dissociation and K_p for the dimer dissociation constant at pressure p. The dissociation constant at atmospheric pressure (K_0) can be determined by linear extrapolation of the plot to 1 bar. Even though the straight line approximation may not represent the physical reality, as will be discussed later, it was justified by the good linear fit

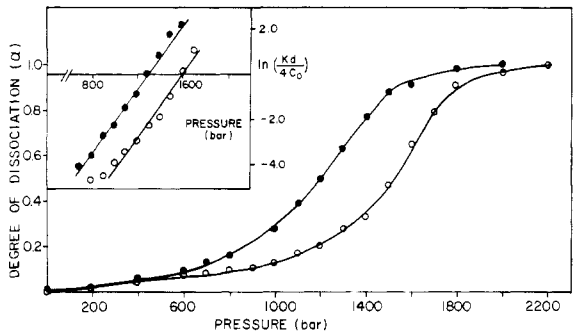

FIGURE 2: Degree of dimer dissociation of holo β_2 vs. pressure as determined by the center of spectral mass of intrinsic fluorescence (eq 2) in standard buffer medium at 25 °C. Excitation wavelength was 280 nm, and emission was monitored from 290 to 420 nm: (○) 12 μM holo β_2: (●) 1.2 μM holo β_2. Inset: Plot of ln ($K_{dp}/4C_0$) vs. pressure for (○) 12 μM holo β_2 and (●) 1.2 μM holo β_2.

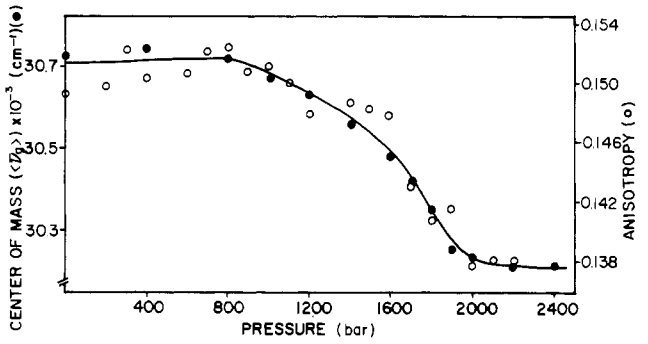

FIGURE 3: Pressure dissociation of reduced β_2 dimer at 2 μM concentration by measurements of intrinsic fluorescence as in Figure 2 (●) and by measurements of anisotropy (eq 3) of 2 μM 2,5-DNS conjugate (○) with excitation at 380 nm and emission viewed through a Corning 3-72 filter and a 2 M sodium nitrite filter.

Table I: Standard Association Volumes, dV^0, and Dissociation Constant at Atmospheric Pressure, K_0, of β_2 Tryptophan Synthase Dimer

β_2 form	method[a]	concn (μM)	dV^0 (mL/mol)	K_0 (M)
holo, 25 °C	A	1.2	169	4.2×10^{-10}
	A	12	174	3.3×10^{-10}
holo, 4 °C	A	2.0	189	3.6×10^{-9}
reduced, 25 °C	B	2.0	151	6.3×10^{-11}
	A	2.0	152	6.9×10^{-11}
apo, 25 °C	B	2.0	168	2.2×10^{-9}
	A	2.0	184	1.1×10^{-9}

[a] Method A, measurement of center of mass of fluorescence spectrum; method B, fluorescence polarization of dansyl conjugate.

($r > 0.998$) found in this case. For the plot in the figure dV^0 = 160 mL/mol and $K_0 = 4 \times 10^{-10}$ M. The subnanomolar value for the dissociation constant of holo β_2 dimer precluded its direct determination by a dilution curve. Increasing the protein concentration by 1 order of magnitude resulted in an increase in the pressure at mid-dissociation ($p_{1/2}$) of 330 bars, very close to what is theoretically expected for a dimer–monomer equilibrium with the dV_0 = 160 mL/mol (Paladini & Weber, 1981; Weber, 1986). As shown in Table I, K_0 and dV_0 values were very similar for the two concentrations.

Figure 3 shows the pressure-induced dissociation for the β_2 subunit after reduction of the cofactor. The degree of dissociation was determined by the center of spectral mass and by polarization of the fluorescence of dansyl-labeled protein, a method that directly measures the average volume of the protein particles (Paladini & Weber, 1981). It was not possible to use dansyl-labeled intact holo β_2 for polarization measurements because of the significant contamination of the

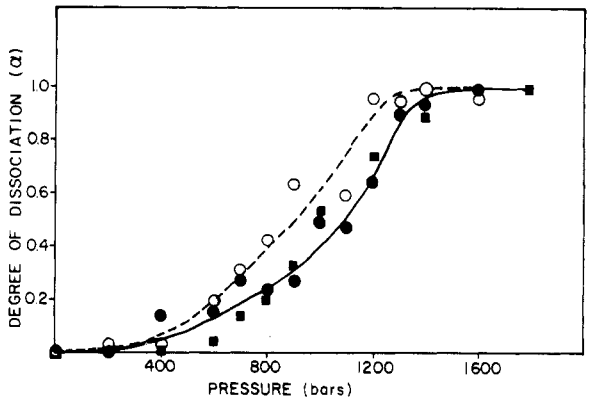

FIGURE 4: Plot of degree of dimer dissociation of apo β_2 vs. pressure at two concentrations, 2 μM (●, ■) and 0.2 μM (○). Measurements by polarization of 2,5-DNS conjugate (●, ○) and by center of mass of intrinsic fluorescence (■). Other conditions as in Figure 2.

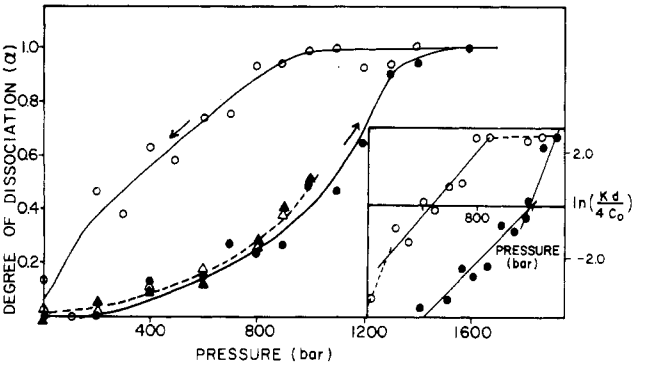

FIGURE 5: Hysteresis effects in the pressure dissociation of apo β_2. The continuous lines are for increasing pressure (●) to complete dissociation followed by decreasing pressure (○) to 1 bar. The dashed lines are for increasing pressure (▲) to about 50% dissociation followed by decreasing pressure (△) to 1 bar. Protein concentration was 2 μM. Values of degree of dissociation were determined from measurements of polarization of 2,5-DNS conjugates of apo β_2. Other conditions as in Figure 4. Inset: Plot of ln ($K_p/4C_0$) vs. pressure for increasing pressure (●) and decreasing pressure (○).

dansyl fluorescence by that of pyridoxal-P bound to the enzyme. Reduction of pyridoxal shifted both its excitation and emission spectra to the blue, so that they no longer overlapped the corresponding dansyl spectra. A close agreement of the results obtained by the polarization and center of mass methods was found (Figure 3; Table I). The dissociation constant, 6.6 $\times 10^{-11}$ M, was significantly smaller than that obtained for the untreated holo enzyme (Table I), but the volume change was not very different. As in the holoprotein, a 10-fold decrease of the protein concentration lowered $p_{1/2}$ by the expected magnitude.

Pressure Dissociation of Apo β_2 Dimer. Dansyl polarization and tryptophan spectra were used to follow the pressure dissociation of apo β_2 dimer. Figure 4 shows the pressure dissociation curves at two concentrations of apo β_2 differing by a factor of 10. The dissociation profile reveals many differences with the holoprotein: The apoprotein was more readily dissociated by pressure (Figure 4), in agreement with measurements of enzyme activity after release of pressure (Seifert et al., 1985). The dissociation curve for the apo β_2 was very asymmetric; while 600 bars was necessary to increase the degree of dissociation from 0.1 to 0.5, only 200 additional bars was required to reach a dissociation of 0.9 (Figure 4). The high degree of asymmetry is also evident in the departure from linearity in the plots of ln ($a^2/(1 - a)$) vs. pressure (inset of Figure 5). Below 50% dissociation the slope, and consequently dV^0, were approximately equal to those found for the holo and

reduced protein, but at higher pressures the slope markedly increased. As dV^0 is, in principle, a function of pressure, the asymmetry in the effect of pressure can be explained either by an almost discontinuous increase in dV^0 with pressure or by a large decrease in the affinity between the monomers at the higher degrees of dissociation (Xu & Weber, 1982; Weber, 1986). A change in dV^0 with pressure requires a difference in compressibility between products and reactants, but as originally pointed out by Bridgman (1931), the decrease in compressibility with pressure may be expected to minimize the departure from linearity exhibited in plots of the work of compression vs. pressure. The magnitude of the effect observed in the apoprotein is then much too large to be due to difference in the overall compressibilities of monomer and dimer. The other possible explanation is simpler and implies the loss of a considerable fraction of the free energy of association after 50% dissociation. We thus conclude that in this case, as in the pressure dissociation of lactate dehydrogenase (King & Weber, 1986a,b), we are witnessing the effects of a conformational drift that takes place when the monomers become separated from each other and that this effect is large enough in the apo β_2 tryptophan synthase to result in the observed asymmetry of the effect of pressure upon degree of dissociation. The increasing loss in affinity at dissociations higher than 50% is in fact predicted in the theory of the conformational drift offered by Weber (1986). A careful look at Figures 2 and 3 reveals that some degree of asymmetry is also present in the pressure dissociation of both the holo and reduced β_2 dimer. However, it was small enough to allow us to accept the logarithmic plots as bona fide straight lines.

Hysteresis Effects. It was found that the degree of dissociation of apo β_2 measured upon increase of pressure was systematically smaller than the one measured when the same pressure was reached by a gradual decrease after complete dissociation was reached (Figure 5). This hysteretic behavior directly indicates that the subunits lost affinity upon dissociation. The difference in the pressures at midpoint dissociation for the two conditions ($p_{1/2}$(up) and $p_{1/2}$(dn)) was 700 bars. Accordingly the loss in standard free energy of dissociation was

$$(p_{1/2}(\text{up}) - p_{1/2}(\text{dn}))dV^0 = 0.700 \times 160/24.8 = 4.52RT \quad (4)$$

or 2.62 kcal/mol.

The plot in the inset of Figure 5 indicates that the large hysteresis arising from the loss of free energy of association is related to the departure from linearity of the compression curve. In fact, when the pressure was increased to a determined value below the break point in the linearity of the latter curve, the return to atmospheric pressure occurred without appreciable hysteresis (Figure 5) and dV^0 determined from the decompression curve was 140 mL/mol, close to the corresponding value for the linear part of the compression curve (Figure 5; Table I).

Experiments of compression followed by decompression were carried out with holo β_2 subunits following the time protocol used for the apoprotein (Figure 6). A lower degree of hysteresis was found, indicating that the binding of pyridoxal-P stabilizes the protein in respect to the loss of subunit affinity upon dissociation.

Temperature Dependence of the Pressure Dissociation. The compression and decompression curves observed at 4 °C with β_2 holoprotein are shown in Figure 6. Dissociation was facilitated by lowering the temperature. K_0, the dissociation constant at 1 bar, was about 10 times larger at 4 °C (Table I) than at 25 °C. The increased instability at low temperature

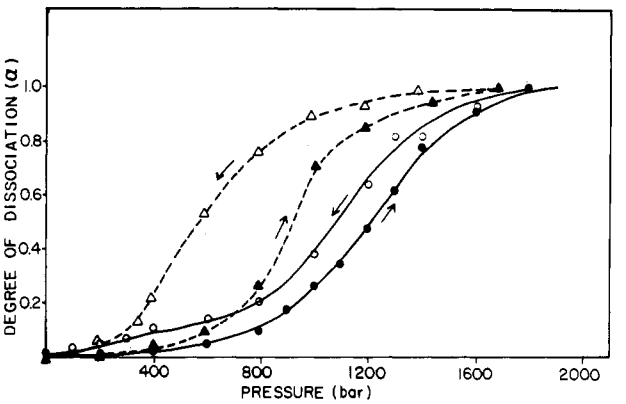

FIGURE 6: Hysteresis effects in the pressure dissociation of holo β_2 at two temperatures. Degrees of dissociation were calculated from measurements of center of spectral mass. Protein concentration was 2 μM. Continuous lines are for increasing (●) and decreasing (○) pressure at 25 °C. Dashed lines are for increasing (▲) and decreasing (△) pressure at 4 °C. All other conditions as in Figure 2.

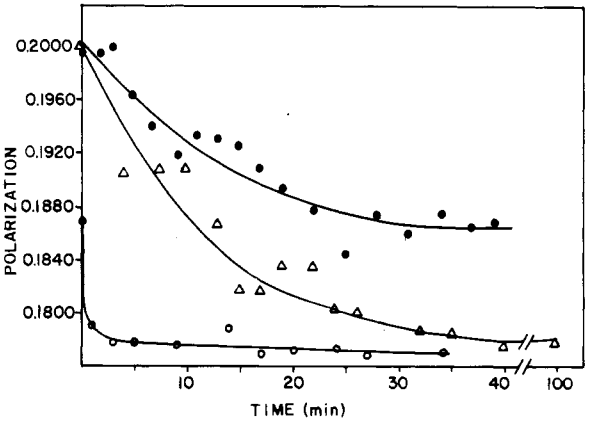

FIGURE 7: Time course of the polarization of 2,5-DNS conjugate of reduced β_2 upon a rapid increase in pressure: (△) increase from 1 to 2.2 kbars; (●) increase from 1 to 1.6 kbars; (○) increase from 1.6 to 2.2 kbars after 40 min at 1.6 kbars. Protein concentration was 2 μM. All other conditions as in Figure 3.

Table II: Loss in Standard Free Energy of Monomer Association, dG^0, Calculated from the Difference in Pressures of Half-Dissociation upon Compression and Decompression, $dp_{1/2}$

β_2 form	$dp_{1/2}$ (bars)	dG^0 (kcal/mol)
apo, 25 °C	700	2.62
holo, 25 °C	150	0.56
holo, 4 °C	350	1.31

is also apparent from the larger degree of hysteresis at 4 °C in comparison to that at 25 °C (Figure 6; Table II). The difference in $p_{1/2}$ for the compression and decompression branches (eq 4) was 350 bars at 4 °C and only 150 bars at 25 °C, indicating that the conformational drift was more pronounced at 4 °C. The decreased affinity and the increased conformational drift act synergistically to increase the instability of the system at the lower temperature.

Time Course of the Association and Dissociation. Figure 7 shows the time profile of the dimer dissociation of holo β_2 dimer as followed by the changes in the fluorescence polarization of a dansyl conjugate. Upon application of 2.2 kbars (triangles) complete dissociation was reached in 40 min as shown by a polarization change equal to that displayed in Figure 3, when the pressure was increased by steps up to 2.2 kbars over a much longer time. If the pressure was raised to only 1.6 kbars (filled circles), the dissociation was 60%, but in both cases the time for achieving half of the polarization

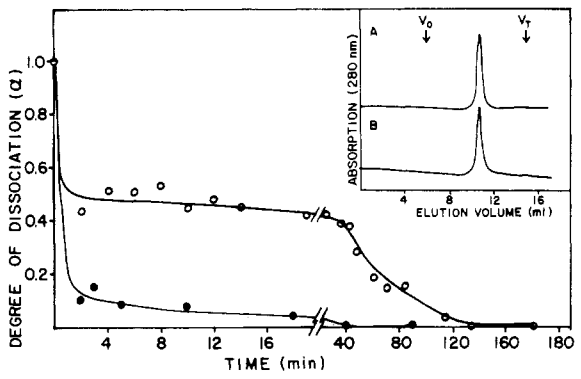

FIGURE 8: Time course of dimer association upon release of pressure. Degrees of dissociation were obtained from polarization of 2,5-DNS conjugates of the protein: (●) 2 μM 2,5-DNS-reduced holo β_2; (○) 0.5 μM 2,5-DNS apo β_2. All other conditions as described in Figure 3. Inset: size HPLC of holo β_2 with the TSK G-3000 SW column eluted with 0.05 M Bis-tris-propane, 0.2 M sodium acetate, 1 mM EDTA, and 0.5mM DTE at pH 7.0. The flow rate was 1 mL/min. In (A) 100 μL of 1.2 μM holo β_2 was injected. In (B) the same sample was incubated at 1.6 kbars for 40 min, and 100 μL was injected immediately after release of pressure.

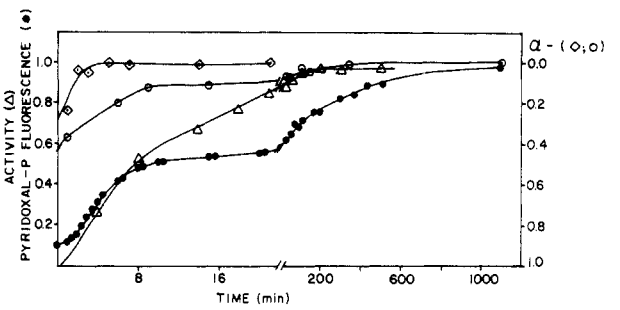

FIGURE 9: Time course of recovery of monomer association, enzymatic activity, and pyridoxal-P fluorescence upon release of pressure: (◇) return of polarization of 2 μM 2,5-DNS-reduced β_2 incubated for 40 min at 2.2 kbars; (○) return of center of spectral mass of 2 μM holo β_2 incubated for 40 min at 1.8 kbars; (△) return of enzymatic activity of 2 μM holo β_2 incubated for 40 min at 1.8 kbars; (●) return of total intensity of pyridoxal-P fluorescence of 2 μM holo β_2 incubated for 40 min at 1.8 kbars. The abscissa is the time after release of pressure.

change was similar (approximately 12 min). From this similarity we conclude that the rate of dissociation observed after a sudden rise above atmospheric pressure must be the rate of dissociation at *atmospheric pressure*, $k_- = 1.5 \times 10^{-3}$ s^{-1}. When, after 1 h at 1.6 kbars, the pressure was further raised to 2.2 kbars (open circles), equilibration was virtually completed within the first measurement ($t < 1$ min). From the large difference of this dissociation rate with the rates observed when the solution was originally at atmospheric pressure, we conclude that the bulk of the increase in the dissociation constant with pressure is due to an increase in the rate of dissociation. From the estimated rate of dissociation at atmospheric pressure and $K_0 = 6.6 \times 10^{-11}$ M, we derive a rate constant for association $k_+ = 2.3 \times 10^7$ s^{-1} M^{-1}. At micromolar concentration we expect a half-time for association $t_+ = 1/(2k_+C) < 1$ s and therefore immediate association on release of pressure, even if there is some loss of monomer affinity owing to the conformational drift. When, after 90 min at a pressure of 2.2 kbars, the solution was rapidly returned to atmospheric pressure, the association was indeed faster than the time resolution of the method. The first measurement, after some 2 min, indicated that more than 90% of the protein had already associated. The fast association of holo β_2 subunit was further verified by size chromatography in an HPLC column. A sample of holo β_2 was fully dissociated by pressure and injected into the sizing column 2 min after release of pressure (inset of Figure 8). It eluted in the position of the untreated control, corresponding to a particle of 90 kDa as determined from calibration with known globular proteins. In the case of the apo β_2 dimer, in which the loss of subunit affinity was so much larger, 10–15% of dissociated protein was found at atmospheric pressure after decompression (Figure 5). In order to investigate further the relation between association and conversion of drifted to native dimer, a 0.5 μM apoprotein solution was subjected to a pressure of 1.6 kbars for 30 min, and then the pressure was released to 1 bar (Figure 8). The degree of dissociation immediately dropped to 0.45. From the rapidly reached plateau, one could conclude that equilibrium was attained, but on continued observations the system revealed a second stage of slow association leading to a negligible value of degree of dissociation after several hours. The striking difference in time course between the processes at the two protein concentrations leaves no doubt about the causes. The fast association component was, nearly, a diffusion-controlled process where drifted monomers formed drifted dimer. As the dissociation constant increased by about 2 orders of magnitude owing to the conformational drift of the monomers, at 0.5 μM concentration 40% of the protein would not immediately associate at atmospheric pressure. The subsequent association was rate-limited by the slow conversion of drifted to native protein, which may be presumed to take place exclusively in the associated dimers. It should be pointed out that the rapid attainment of a negligible dissociation at atmospheric pressure does not mean that the system had already recovered its original free energy of association, but that the affinity of the drifted species was sufficient to permit virtually complete association at 1 bar at the concentration employed.

Recovery of Functional Properties. Figure 9 shows the recovery of different properties of tryptophan synthase β_2 subunit. It has already been shown that the polarization of the intrinsic protein fluorescence of β_2 returned to the original value very quickly and did not change in 12 h, indicating that the dimeric structure was immediately recovered. The average energy of the intrinsic fluorescence also returned on decompression to the extent of 80–85%, and the remainder was recovered though much more slowly. The slower recovery further reflects the conversion of drifted to native dimer through its effects upon the microscopic environment of the tryptophan residue. The recovery of activity was much slower than the reassociation (Figure 9), giving independent support to the view that an associated dimer with altered conformational properties is obtained upon decompression.

The fluorescence intensity of pyridoxal-P was also monitored after release of pressure: As shown in Figure 9, 50–60% of it returned with a time constant similar to the recovery of activity, therefore well after the association of the monomers. The remaining 40–50% returned very slowly ($t_{1/2} \approx 300$ min). It is noteworthy that the dimer did not need to recover the second component of pyridoxal-P fluorescence to become fully active. Either further recovery was brought about rapidly by the α subunits and the substrates in the assay medium or it was not necessary for activity, being only the reflection of functionally unimportant isomerizations in the protein.

Discussion and Conclusions

Seifert et al. (1984) measured the enzymic activity of solutions of the β_2 dimer of tryptophan synthase kept previously under pressure for a standard period. They calculated degrees of dissociation *under pressure* on two assumptions: (1) Reassociation was supposed slow in comparison with the times necessary to open the pressure cell and make the activity

measurements at atmospheric pressure. (2) The solutions were supposed to contain no other species besides active dimers and inactive monomers. We find that neither of these assumptions is valid. Release of pressure is followed by association within the minute or two required for the measurements (Figure 8) and the persistence of an altered β_2 dimer is shown by the recovery of the initial molecular size without the concomitant return of activity and pyridoxal-P fluorescence (Figure 9). The presence of an inactive dimer that relaxes slowly to the active form precludes the interpretation of the β_2 subunit dissociation as a single equilibrium; whatever model is postulated, the description of the equilibria between dimer and monomers must include *at least two* conformationally different forms. We refer to these as the "native" and "drifted" forms, although we are aware that the drifted form is almost certainly a heterogeneous population of unknown distribution. While a conformational drift of the subunits of an oligomer has to be invoked to explain the results of experiments at atmospheric pressure [dilution curves, Shore and Chakrabarthy (1976) and Xu and Weber (1982); cold inactivation, King and Weber (1986b)], the hydrostatic pressure is unique in accessing directly the loss of free energy of association and in permitting the ready study of the properties of the drifted dimer. Oligomers with altered properties have been repeatedly observed: Irias et al. (1969) reported that the reassociation upon warming of cold-dissociated pyruvate carboxylase involved the intermediate formation of an inactive tetramer. Goldenberg and King (1982) describe an intermediate already associated, but not completely folded, in the in vivo subunit assembly of tail spike of endorhamnosidase of bacteriophage P22. In tryptophan synthase β_2 subunit itself, Blond and Goldberg (1985) reported an inactive dimer intermediate after removal of the denaturing conditions. However, in none of the three last cases was the subunit affinity of the altered oligomer determined.

Since all the inferences about the system come from the determination of the degree of dissociation for a given condition, this must be direct and precise. Two different spectroscopic methods were used to measure the degree of dissociation. One of them, the polarization of dansyl covalently attached to the protein, measures the rotational diffusion of the protein particles and therefore their size (Weber, 1952a,b). The other method we employed, the spectral distribution of the intrinsic fluorescence, is extremely sensitive, allowing detection of differences in the center of spectral mass smaller than 10 cm^{-1} (<0.1 nm) (Royer, 1985). The good agreement found between the degrees of dissociation obtained by the two methods and the concentration dependence observed in the respective dissociation curves strongly corroborates the conclusion that both reveal the dissociation of the protein.

Recently, Blond and Goldberg (1985) identified an inactive dimer intermediate on the folding pathway of β_2 and nicked β_2 subunit. After dilution of the denaturing agent, they found a slow renaturation of the monomer followed by a fast dimerization and then by isomerization of the dimer to the active form. In our case, the first slow isomerization step was lacking, in all probability because the monomers do not undergo extensive unfolding upon pressure dissociation, and consequently reassociation to dimers takes place readily when the pressure is released. Studies on single-chain proteins indicate that a pressure of 2.5 kbars should have no serious effect upon the conformation (Brandts et al., 1970; Hawley, 1971; Zipp & Kauzmann, 1973; Li et al., 1976a,b; Visser et al., 1977; Heremans & Wong, 1985). The slow return of activity after decompression is not exclusive of tryptophan synthase β_2 subunit. The activity of lactate dehydrogenase returns very slowly, and depending upon the magnitude and duration of the applied pressure, the recovery may take days (Mueller et al., 1981, King & Weber, 1986a).

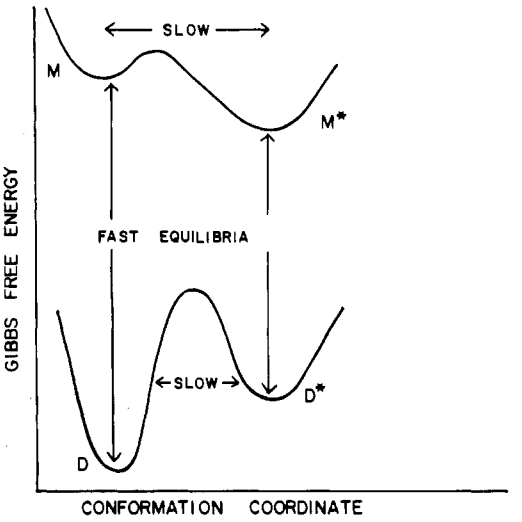

FIGURE 10: Free energy diagram of the conformational drift of tryptophan synthase β_2 subunit.

Adachi and Miles (1974) reported that the β_2 subunit can be obtained in two forms, a low- and a high-activity form, and that the loss of activity can occur during purification or storage. It is possible that the conversion of high activity β_2 dimer to low-activity form arises from a conformational drift during some step of the purification procedure.

The hysteresis of the pressure curves indicates that the drifted dimer has decreased affinity between subunits. The origin of the hysteresis may be understood by means of the free energy diagram (Figure 10) proposed by King and Weber (1986b). This involves four characteristic forms: native and drifted monomer, respectively M and M*, and native and drifted dimer, respectively D and D*. The second-order equilibria ($2M \rightleftarrows D$ and $2M^* \rightleftarrows D^*$) are assumed to be much faster than the first-order conversions $M \rightleftarrows M^*$ and $D \rightleftarrows D^*$. Evidence for these conditions is provided in Figures 7–9. In the description of the dissociation equilibrium of a dimer subjected to conformational drift proposed by Weber (1986), the ratio $[M^*]/[M]$ is not a constant but a function of the degree of dissociation of the system: From a negligible value at $a \rightarrow 0$ it increases slowly up to $a = 0.5$ and becomes much larger than unity for values of a between 0.5 and 1 [Weber, 1986 (Figure 5)]. When dimers are formed after pressure release, we expect the transition $D^* \rightarrow D$ to become progressively slower as the proportion of drifted monomers present before reversal of pressure increases. This characteristic provides a rational explanation for the observation that the β_2 apoprotein shows no hysteretic behavior nor appreciable loss of free energy of association if the dissociation is kept below 0.5, but both phenomena are very pronounced if complete dissociation is reached. The free energy diagram of Figure 10 explains the slow conversion of drifted into native dimer as the result of a large activation energy barrier, in agreement with the experimental observation of the increased instability of the system at low temperature. As the conversion of drifted to native dimer is very slow (Figures 8 and 9), the decompression curve represents essentially the equilibrium between the drifted forms, $2M^* \rightleftarrows D^*$.

An iterative simulation of the conformational drift in terms of the coupled equilibria of Figure 10 was done by Weber (1986). The incomplete equilibration at each pressure and

the ensuing hysteresis were simulated by reducing the number of iterations below that required for equilibration. The difference $dp_{1/2}$ in the pressures of half-dissociation of the compression and decompression curves presented a gaussian-like dependency on the logarithm of the number of iterations [Figures 7 and 8 of Weber (1986b)], and it was found that the maximum $dp_{1/2}$ corresponded to 70% of the loss of free energy of association due to the conformational drift. Thus, the experimental $dp_{1/2}$ values and the derived losses of free energy, dG, presented in Table II are to be considered as lower limits. It should be pointed out that the assumption of two coupled equilibria is the simplest postulation and one that is not expected to accommodate all the observations. For example, the bimodal return of the pyridoxal-P fluorescence indicates that the system is more complicated than the model presented in Figure 10.

The questions about the biological relevance of the conformational drift are certainly the most important to be investigated. The larger conformational drift that follows the removal of pyridoxal-P from the β_2 dimer points to the importance of ligand binding in the stabilization of the free energy of subunit association and therefore of the quaternary structure. The affinity of the apoprotein was about 10 times lower than that of the holoprotein, and the loss of free energy of association in the apo form was large enough to permit detection of free monomer after a cycle of dissociation–association. Zetina and Goldberg (1980) found that pyridoxal-P protected the β_2 subunit against thermal inactivation, and hybrid dimers were also stabilized, indicating an intersubunit mechanism. The direct involvement of pyridoxal-P in the oligomer stabilization was also observed in tryptophanase, where it limited the tetramer–dimer dissociation induced by low temperature (Morino & Snell, 1967).

It is tempting to generalize that the loss of subunit affinity due to conformational drift is rather undesirable and that defense mechanisms exist to limit the latter or to overcome its consequences. Their identification should provide an interesting subject for further studies.

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