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RECONSTITUTION OF THE ISOLATED β_2-SUBUNIT OF TRYPTOPHAN SYNTHASE FROM *ESCHERICHIA COLI* AFTER DISSOCIATION INDUCED BY HIGH HYDROSTATIC PRESSURE

EQUILIBRIUM AND KINETIC STUDIES

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The isolated β_2-subunit of *Escherichia coli* tryptophan synthase can be reversibly dissociated into enzymically inactive monomers under high hydrostatic pressure. Deactivation at 1.5 kbar which shows a half-time of 11 min (rate constant $k = 10^{-3}$ s^{-1}) is paralleled by dissociation with a small lag phase of about 5 min. Pressure release leads to 95 ± 5% recovery of specific activity and complete restoration of the hydrodynamic and spectral properties which specify the native dimer. Over the concentration range 1–100 μg/ml (0.02–2.3 μM) the kinetics of reactivation can be fitted by one apparent first-order rate constant ($k = 6.5 \pm 0.6 \times 10^{-4}$ s^{-1}, half-time = 17.5 min). The reconstitution of catalytic activity is paralleled by alterations in tryptophan fluorescence at 327 nm, thus presenting direct evidence for conformational changes in the direct vicinity of the active center ($k_1 = 1.9 \times 10^{-3}$ s^{-1}, $k_2 = 6.5 \pm 0.6 \times 10^{-4}$ s^{-1}). On the other hand, a definite mechanism of reactivation requires the association of the refolding monomers to be included. The kinetics of dimerization have been followed via hybridization between native and chemically modified β-chains, yielding an apparent first-order rate constant of $6.3 \pm 0.6 \times 10^{-4}$ s^{-1}. As a consequence, we propose a sequential uni–uni–bimolecular mechanism, which is characterized by a minimum of two conformational changes in substantially structured monomers followed by a fast dimerization reaction to yield the active β_2-subunit.

1. Introduction

The isolated β_2-subunit of the tryptophan synthase $\alpha_2\beta_2$-bienzyme complex from *Escherichia coli* (L-serine hydro-lyase (adding indoleglycerol-phosphate), EC 4.2.1.20) catalyzes the final step in tryptophan biosynthesis:

Indole + L-serine → L-tryptophan + H_2O. (1)

This irreversible condensation may be formally regarded as a partial reaction of the overall turnover catalyzed by the native $\alpha_2\beta_2$ complex:

Indoleglycerol phosphate + L-serine → L-tryptophan + D-glyceraldehyde 3-phosphate + H_2O. (2)

Pyridoxal 5′-phosphate is required as coenzyme for both reactions. The β_2-subunit, which represents an extremely stable dimer, spontaneously associates with the corresponding α-chains to form the tetrameric enzyme. The simultaneous increase in catalytic efficiency and affinity for substrates which accompanies this heterologous interaction is thought to be caused by mutually induced conformational changes in the α- and β_2-subunits [1–3].

After the folding and association of intrinsic structural domains within the peptide chain, the specific association of subunits to yield the native quaternary structure is the ultimate stage in the self-organization of proteins. Since the in vitro reconstitution of monofunctional oligomeric enzymes has been analyzed in great detail [4], it would be of interest to compare the compiled results with corresponding data for components of

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multienzyme complexes. In a recent paper, we therefore investigated the refolding and reactivation of the β_2-subunit of tryptophan synthase after denaturation in acidic guanidine hydrochloride [5]. However, refolding of the more or less randomized peptide chains – as obtained from denaturation in acid, urea or guanidine hydrochloride—to a structured monomeric intermediate competes with the formation of inactive aggregates [6], leading to low yields of reactivation at higher protein concentrations. This undesired side reaction may be suppressed by applying high hydrostatic pressure to dissociate the enzyme [7,8] into monomers in which a comparatively high degree of structural information is conserved.

In this work we investigate the pressure-induced deactivation and dissociation as well as the reconstitution of the isolated β_2-subunit of tryptophan synthase by equilibrium and kinetic experiments. The results are interpreted on the basis of a uni–uni–bimolecular mechanism.

2. Materials and methods

Tryptophan synthase β_2-subunit was purified from the A2/F'A2 mutant strain of *E. coli* (kindly donated by Drs. C. Yanofsky and I.P. Crawford) and stored frozen at −75°C according to ref. 9.

Pyridoxal 5'-phosphate was obtained from Serva (Heidelberg), tris(hydroxymethyl)aminomethane (Tris) and dithioerythritol from Roth (Karlsruhe). All other chemicals were of A grade purity from Merck (Darmstadt). Quartz-bidistilled water was used for making up solutions.

If not stated otherwise, all experiments were performed in buffer A: 0.1 M Tris buffer (pH 7.8), 0.1 M NaCl, 10 mM dithioerythritol, 0.5 mM EDTA, 0.84 mM pyridoxal 5'-phosphate. (Buffer B: 0.1 M potassium phosphate buffer (pH 7.8), 0.2 mM dithioerythritol, 0.5 mM EDTA, 40 μM pyridoxal 5'-phosphate.) Apo-β_2-subunit was prepared by passing holoenzyme through a small band of 0.5 M hydroxylammonium chloride on a Sephadex G-25 column (0.8 × 25 cm) equilibrated with buffer A (without pyridoxal 5'-phosphate). The concentration of the β_2-subunit was determined according to Bradford [10]. Enzymatic activity was measured as described by Faeder and Hammes [11] using a Bausch & Lomb spectrophotometer. The activity of the freshly prepared holoenzyme was about 3800 Yanofsky units/mg or 13 I.U./mg. One Yanofsky unit is defined as the amount of enzyme that catalyzes the conversion of 0.1 μmol indole to L-tryptophan per 20 min at 37°C.

Absorbance, fluorescence emission and circular dichroism were measured with a Zeiss DMR 10 spectrophotometer, a Hitachi-Perkin-Elmer MPF44A spectrofluorimeter and a Roussel-Jouan Dichrographe II, respectively.

Descending gel chromatography on Sephacryl S-200 was performed at 4°C using Pharmacia columns (Freiburg) and a Gilson Minipuls 2 peristaltic pump (Abimed, Düsseldorf) with flow rates of 10 ml/h. High-pressure quenching experiments for the determination of pressure-dependent dissociation were performed with an autoclave which has been described elsewhere [7]. 1,2-Ethanediol was used as pressure-transmitting liquid because of its low compressibility, which keeps pressure-induced temperature changes in the autoclave to a minimum. The pressure-generating equipment was identical with that described in refs. 12 and 13. Enzyme samples of the indicated concentrations were incubated at 10°C in buffer A for defined time intervals. After pressure release the protein solution was immediately removed and the enzymatic activity was measured under normal pressure at 25°C as described above. In order to prevent reactivation during the test, the experimental conditions for measuring reactivation kinetics had to be altered slightly by lowering the temperature from 37 to 25°C and by adding 10 μg/ml trypsin (TPCK-treated) to the test medium [14].

Hybridization experiments were performed in order to determine the degree of pressure-induced dissociation and the reassociation following pressure release [27]. For this purpose, a chemically modified β_2-subunit was prepared in which the internal aldimine between pyridoxal 5'-phosphate and the ϵ-aminolysyl side chain 86 was reduced with $NaBH_4$ [15,16]. To follow the kinetics of dissociation, equal amounts of native and modified β_2-subunit were incubated together for the

indicated time intervals. Hybridization of the species dissociated thus far occurred under normal pressure. The reassociation kinetics of the completely dissociated enzyme were measured by taking aliquots at defined times from samples (holo-β_2-subunit alone) reconstituting under normal pressure (10°C) which were subsequently quenched by immediate addition of a 5-fold excess of freshly pressure-dissociated, modified β-subunit monomers in buffer A containing 75 μM pyridoxal 5'-phosphate. The degree of hybridization was analyzed via polyacrylamide gel electrophoresis under native conditions according to the general procedures described previously [17,18,28].

Denaturation and renaturation of the enzyme were detected by recording the fluorescence emission of Trp-176 in the β-chain at 327 nm (excitation at 280 nm) in buffer A containing 75 μM pyridoxal 5'-phosphate.

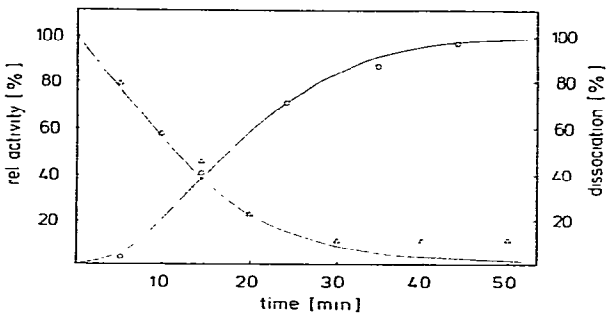

Fig. 1. Kinetics of deactivation and dissociation of the isolated β_2-subunit of tryptophan synthase during exposure to 1.5 kbar at 10°C. Protein concentration, 100 μg/ml (2.3 μM); enzymic test as described in section 2. (△) Deactivation, (○) dissociation measured via hybridization with chemically modified, reduced β-chains.

3. Results

3.1. Equilibrium measurements

3.1.1. Deactivation, denaturation and dissociation

For reconstitution experiments the long-term stability of the respective enzyme is of crucial importance. Consequently, optimum solvent conditions for deactivation and dissociation of the isolated tryptophan synthase β_2-subunit into monomers have to be established in order to minimize interference between reconstitution and uncontrolled decay of the enzyme. For instance, it is known that the activity of the β_2-subunit depends strongly on the presence of specific ions and of SH-group-protecting agents [24]. A remarkable increase in stability of the β_2-subunit can be accomplished by adhering strictly to anaerobic conditions (i.e., flushing oxygen out of buffers with an excess of purified nitrogen and withdrawing samples for the test assay exclusively under a nitrogen atmosphere). Taking these precautions the specific activity of the enzyme in buffer A remains essentially constant for at least 100 h.

After 15 min incubation in buffer A at 2 kbar and 10°C complete deactivation of the enzyme can be observed. This reaction is accompanied by a significant increase in the fluorescence quantum yield of Trp-176, indicating substantial denaturation of the protein (table 1). Beyond that, the β_2-subunit is effectually dissociated into monomers as shown indirectly by hybridization between native and chemically modified β-chains (fig. 1).

3.1.2. Reassociation, renaturation and reactivation

After pressure release, reassociation, renaturation and reactivation take place to an extent of at least 95%. Under the given conditions, the high reactivation yields obtained are constant within the concentration range under consideration (2.3 $\mu M > c > 23$ nM). In order to characterize the final product of reactivation the reconstituted and the native enzyme were compared. Applying gel chromatography both species are characterized by congruous elution profiles on Sephacryl S-200. The ultraviolet absorption, intrinsic fluorescence emission, far-ultraviolet dichroic absorption and Michaelis constants (K_m) for indole and L-serine coincide for the native and the reactivated enzyme within the range of error of less than 5% (fig. 2, table 1).

3.2. Kinetic measurements

The consequent accordance in the physical characterization of the native and denatured state

Table 1

Characterization of the β_2-subunit of tryptophan synthase in its native, denatured and reactivated states

Enzyme	M_r [a]	Specific activity (%)	F_{rel}	K_m (indole) [b] (mM)	K_m (L-serine) (mM)
Native	86000	100	100	0.019	0.36
Denatured	–	0	197 [c]	–	–
Reactivated	86000	≥95	104	0.020	0.37

[a] Measured by gel chromatography on Sephacryl S-200.
[b] Measured at 37°C according to ref. 11.
[c] Extrapolated from the fast kinetic phase in fig. 5 at $t = 0$ (half-filled circle in fig. 5).

of the enzyme as well as the concentration-independent high yield of reactivation provide ideal conditions for kinetic measurements of the break-up and the reconstitution of the β_2-subunit. The solid lines in fig. 3 illustrate the time-dependent deactivation of the enzyme under different pressures. The deactivation reaction at the given pressure values occurs far from equilibrium. Moreover, as shown below, the reactivation of the enzyme is apparently governed by pseudo first-order kinetics. Therefore, the corresponding volume of activation, $\Delta V^{\ddagger} = -100 \pm 5$ ml/mol, can be calculated from the individual rate constants [7].

Evidently, the process of deactivation is paralleled by dissociation with a small lag phase of about 5 min. The rate constants at 1.5 kbar are $k = 10^{-3}$ s^{-1} for deactivation and $k = 1.2 \times 10^{-3}$ s^{-1} for dissociation (fig. 1).

In the essentially irreversible process of reconstitution after rapid pressure release, first-order conformational changes and a second-order association reaction are expected as elementary steps. The interplay of the relative rate constants of these processes which influences the overall kinetics of the recovery of native structure and enzymatic activity is of crucial significance for the determina-

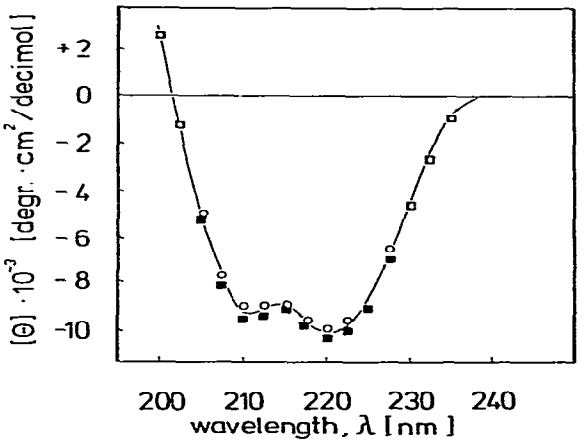

Fig. 2. Comparison of the far-ultraviolet dichroic absorption for the native and the renatured β_2-subunit. 100 μg β_2-subunit/ml buffer B containing 20 μM pyridoxal 5'-phosphate. Denaturation for 20 min at 2.0 kbar (10°C). (○) Ellipticity of the native holo-β_2-subunit, (■) renatured enzyme under identical conditions.

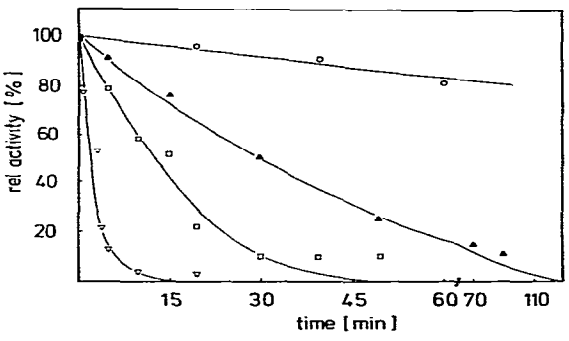

Fig. 3. Determination of the activation volume, $\Delta V^{\ddagger} = -RT\, d(\ln k)/dp = 100 \pm 5$ ml/mol, for the deactivation of the β_2-subunit of tryptophan synthase. Time course of the loss of enzymic activity of 100 μg β_2-subunit/ml buffer A at 10°C during exposure to: (○) 1.0 kbar, (▲) 1.3 kbar, (□) 1.5 kbar and (△) 2.0 kbar.

tion of the underlying mechanism of reconstitution. The regaining of the dimeric quaternary structure of the enzyme has been followed via hybridization as shown in fig. 4.

In order to terminate immediately the dimerization of the enzyme after defined time intervals, the quencher, i.e., the dissociated, chemically modified β-protein, has to be present in an at least 10-fold molar excess. This means that under the given conditions an initial concentration of at least 1 mg modified subunit/ml is required. On the other hand, concentrations of the modified β-chain higher than 0.5 mg/ml show a strong tendency to form unspecific aggregates under the influence of high hydrostatic pressure. Thus, the actual concentration of residual native monomers in a kinetic hybridization experiment has to be allowed to drop considerably below 50 μg/ml in order to obtain relevant data. Considering this limitation, the resulting progress curve is characterized by a half-time of 17.5 $\pm$ 2 min.

Although the recovery of the original dimeric structure of the β_2-subunit represents a bimolecular reaction, the observed process shows no concentration dependence within the range 2.3 $\mu M \geqslant c \geqslant$ 1.15 μM; it can be described according to $\Delta\beta_2 = \beta_2^o \exp(-kt)$ where $\Delta\beta_2$ is the deviation of the observed amount of reassociated enzyme from the final equilibrium value. The corresponding apparent first-order reaction rate constant $k = 6.3 \pm 0.6 \times 10^{-4}$ s^{-1}. This may be taken as evidence that the process of dimerization is governed by a rate-determining first-order conformational change.

The kinetics of renaturation of the enzyme can be measured via changes in the fluorescence emission of the protein at 327 nm. Obviously, the progress curve is biphasic as shown in fig. 5. The predominant part of the kinetic trace can be fitted by one apparent first-order rate constant, $k = 6.5 \pm 0.6 \times 10^{-4}$ s^{-1}, as taken from a semilogarithmic plot of the data (inset, fig. 5). The data are then subtracted from the slow exponential and what remains is used to estimate the fast exponential. Since the two half-times of the two exponential processes merely differ by a factor of 3 this method is not accurate and yields only an approximative

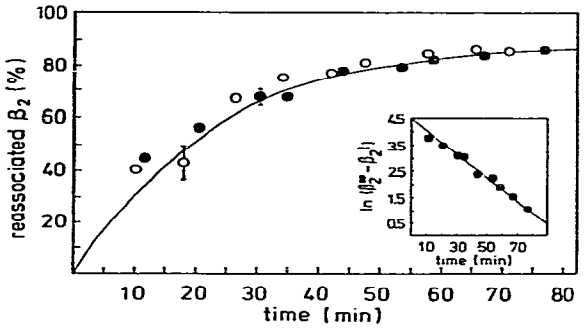

Fig. 4. Kinetics of dimerization of the β_2-subunit after exposure to high hydrostatic pressure. Dissociation of enzyme in buffer A for 20 min at 2.0 kbar (10°C) followed by reassociation measured via hybridization under quenching conditions with 500 μg dissociated, reduced β-chains/ml. Enzyme concentrations: (●) 100 μg/ml, (○) 50 μg/ml. The semilogarithmic plot in the inset (100 μg/ml β_2-subunit) gives $k = 6.3 \pm 0.6 \times 10^{-4}$ s^{-1}. The solid curve was calculated with the given rate constant.

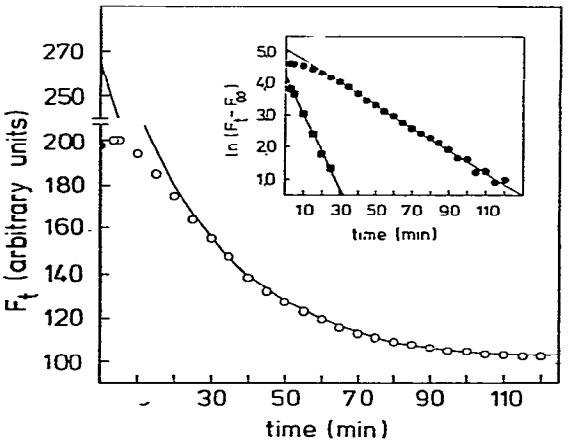

Fig. 5. Kinetics of renaturation of the β_2-subunit of tryptophan synthase. The intrinsic fluorescence of the protein ($\lambda_{ex} = 275$ nm, $\lambda_{em} = 327$ nm) shows a biphasic change during refolding of the protein after exposure to high hydrostatic pressure. (○) 100 μg enzyme/ml buffer B containing 20 μM pyridoxal 5'-phosphate. Deactivation conditions as in fig. 4. The inset shows a semilogarithmic plot of the data resolved into two exponentials. (●) $k_2 = 6.5 \pm 0.6 \times 10^{-4}$ s^{-1}, from which the solid curve was calculated; (■) the preceding rapid phase which is characterized by $k_1 = 1.9 \times 10^{-3}$ s^{-1}; (▶) from table 1.

value of 1.9×10^{-3} s^{-1}. The enzyme contains only one tryptophan residue in position 176 of each β-chain. In the native protein, this aromatic side chain closely interacts with the bound cofactor, pyridoxal 5'-phosphate, as demonstrated by an efficient fluorescence energy transfer between both heterocyclic π-electron systems [19]. Thus, following the kinetics of alterations in the fluorescence emission of Trp-176 provides evidence that the near vicinity of the active center is involved in the observed first-order conformational change.

The reaction order of the rate constant of the recovery of catalytic activity was determined by taking aliquots at defined time intervals and by measuring the time course of reactivation at various enzyme concentrations. The respective activity values are corrected for the reactivation (≈ 3–5%) occurring during the time between pressure release and the first addition of enzyme to the test solution (< 50 s). This inevitable time lapse, however, is small compared to the half-time of the reactivation reaction. As demonstrated in fig. 6, the reactivation of the β_2-subunit is characterized by a single exponential profile which, within the given limits of error, does not depend on enzyme concentration over the range 100–1 μg/ml. This means that in fact a single apparent first-order rate constant, $k = 6.5 \pm 0.6 \times 10^{-4}$ s^{-1}, is adequate to fit the experimental data. The reaction rate determined here is in excellent accordance with the corresponding constant reported for the reconstitution of the β_2-subunit of tryptophan synthase after incubation in 4.5 M guanidine hydrochloride at pH 2.3 [5].

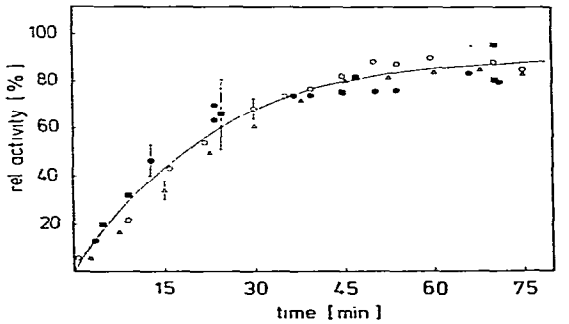

Fig. 6. Kinetics of reactivation of the β_2-subunit of tryptophan synthase after high-pressure incubation. 1 – 100 μg β_2-subunit/ml buffer A were deactivated as described in section 2. (○, △) 100 μg/ml, (●) 10 μg/ml, (□) 1 μg/ml, (———) calculated exponential for $k = 6.5 \pm 0.6 \times 10^{-4}$ s^{-1}.

4. Discussion

The tetrameric $\alpha_2\beta_2$-bienzyme complex tryptophan synthase can be regarded as a well characterized example for the influence of mutual, heterologous interactions between chemically different subunits on catalytic function and structural stability [1,2,20–22]. Even in the absence of the corresponding α-chains, the isolated apo-β_2-subunit of the *E. coli* enzyme has been shown to be a remarkably stable dimer [9,23]. As a consequence, the activation volume for pressure-dependent deactivation of the β_2-subunit is negative but significantly smaller than the values observed for other monofunctional, oligomeric enzymes [7,13]. Thus, the reconstitution of the enzyme under quasi-physiological conditions after pressure release is a rapid process leading to an almost complete restoration of physical and catalytic properties of the protein. Since the exceedingly high yield of reactivation is constant over a concentration range of 2 orders of magnitude, the generation of 'wrong aggregates' [6] may not play an important role during the course of reactivation. From this we conclude that a considerable amount of structural information is conserved in the inactivated protein. Dissociation of the β_2-subunit into monomers is only detected by the indirect method of hybridization between native and chemically modified subunits. Hybrids should be observable after effectual dissociation of the dimers during high-pressure incubation. On the other hand, subunit exchange between unmodified and reduced dimers via encounter complexes is at least a possible alternative mechanism for the generation of hybrids to be considered. A necessary prerequisite for the rapid, spontaneous formation of dimeric hybrids from dimeric pure components would be, however, an appreciably higher stability of such a product of symproportionation. As shown recently [16] this is not the case. In fact, the long-term stability of the hybrid is considerably

lower than that of the pure compound dimers. Thus, we may assume that the inactive protein generated by 15 min incubation at 2 kbar comprises the homogeneous monomer ($M_r = 42942$). A short lag phase has been observed during the kinetics of dissociation of the β_2-subunits into monomers (fig. 1). Since equal amounts of native and modified enzyme are incubated together, it is not clear whether the apparent delay is caused by different dissociation rates of the individual components or is a consequence of conformational changes necessarily preceding the appearance of separated β-chains.

Usually, the enzymatic activity of tryptophan synthase is measured at 37°C [1]. The rate of reactivation, however, is reduced drastically at low temperature [5]. The corresponding reaction enthalpy can be determined from the reaction rates at different temperatures, yielding an estimate of 20 kcal/mol (not shown) [26]. This result might be correlated with the value observed for the *cis-trans* isomerization of proline, which has been supposed to be a rate-limiting step in the attainment of the correct secondary structure during the process of refolding [25]. As a consequence, measuring the kinetics of deactivation and reconstitution of the β_2-subunit at low temperature (10°C) will be much less disturbed by undesired reactivation during the inevitable time lapse between pressure release and the enzymic test.

To measure the regaining of the dimeric quaternary structure via hybridization it is of crucial importance to know the individual association rate constants of the pure components. If the values differ considerably, the slower reacting species has to be surveyed by applying a quenching technique with an excess of the second, faster reacting component. In the case of tryptophan synthase the apparent rate constant for dimerization of the chemically modified subunit is greater by a factor of 2.1 (T. Seifert and P. Bartholmes, unpublished results).

For the concentration range of protein 2.3 μM $\geq c \geq$ 0.02 μM the kinetics of reactivation are independent of concentration. In other words, a defined unimolecular isomerization reaction occurring during the course of reconstitution of the enzyme is rate limiting with $k = 6.5 \pm 0.6 \times 10^{-4}$ s^{-1} over the given range of 2 orders of magnitude. This leads to an estimated minimum value for the second-order rate constant of the expected dimerization reaction of $4 \times 10^5\ M^{-1}\ s^{-1}$, which is in good accordance with the rate of diffusion-controlled encounter of proteins [29]. However, the process of reassociation to the dimeric β_2-subunit as measured by hybridization exactly parallels the progress curve obtained for the reactivation of the enzyme, indicating the rate-limiting participation of the same conformational change in the proper alignment of contact regions of the partially structured monomeric β-chains. The critical isomerization reaction of the refolding β-chains may be directly traced by the described changes in the fluorescence emission of Trp-176. The predominant part of the reaction curve is characterized by a first-order rate constant identical to that mentioned above. A second, faster reaction phase precedes the slow decrease in emission with opposite sign, thereby indicating a complex sequence of structural changes in the near vicinity of the aromatic side chain.

The rate constant characterizing the reconstitution of the β_2-subunit after exposure to high hydrostatic pressure is similar to that obtained from reactivation of the enzyme after complete randomization in 4.5 M guanidine hydrochloride at pH 2.3 [5]. The rate of association of correctly prefolded monomers should be similar, regardless of the structural state of the deactivated enzyme. Therefore, we consider the accurate results of the described hybridization technique as an improvement on the rather qualitative observation of comparatively rapid reassociation obtained from ultrafiltration in the latter case [5]. Consequently, we suggest the following sequential mechanism of reconstitution to be operative:

$$2\mathfrak{M} \xrightarrow[k_1 = 1.9 \times 10^{-3}\ s^{-1}]{\text{uni}} 2M \xrightarrow[k_2 = 6.5 \times 10^{-4}\ s^{-1}]{\text{uni}}$$

$$2M^* \xrightarrow[k_3 \geq 4 \times 10^5\ M^{-1}\ s^{-1}]{\text{bi}} \beta_2$$

where $\mathfrak{M}$, M and M* represent different conformation states of inactive monomers; k_i are the rate constants of the first-order conformation changes and the second-order association step, respectively, as described in the text. The M–M* transition

may be a slow reorientation of the two structural domains F_1 and F_2 of the β-chain as has been supposed recently [30].

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