Isolation and Characterization of Independently Folding Regions of the β Chain of *Escherichia coli* Tryptophan Synthetase†

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ABSTRACT: It had been reported previously that the β_2 subunit of *Escherichia coli* tryptophan synthetase [L-serinehydrolyase (adding indole) EC 4.2.1.20] can be cleaved by trypsin into a nearly functional dimeric protein, the monomer of which consists of two large, nonoverlapping, polypeptide fragments. In the present paper, it is shown that these fragments can be separated after denaturation. Upon removal of the denaturing agent, the isolated fragments spontaneously refold into conformations which, by various physical-chemical criteria, are shown to approximate the conformations of the corresponding fragments associated within the native protein. Furthermore, it is demonstrated that, upon mixing, these renatured fragments reassociate to form the *renatured* nicked protein which, by all the physical and functional criteria used, is indistinguishable from the *native* nicked protein. These results are taken as strong evidence that the isolated fragments can be considered as independently folding regions corresponding to intermediates in the folding of the intact protein.

The mechanisms by which proteins acquire their specific, biologically active, three-dimensional structure are currently studied by a variety of methods: fast kinetics (Baldwin, 1975; Garel et al., 1976), analysis of the properties of the final structure (Levitt and Chothia, 1976; Wetlaufer, 1973), computer simulation of the folding process (Levitt and Warshel, 1975), and chemical (Ristow and Wetlaufer, 1973; Creighton, 1975; Acharya and Taniuchi, 1976) or physical-chemical (Anfinsen, 1973; Schreier and Baldwin, 1976) identification of intermediates in the folding process. Even though they were used on very small proteins (molecular weights below 15 000), these methods are still quite complex and do not appear applicable, in their present state of development, to most proteins which are usually made of much larger polypeptide chains.

In order to account for the folding of larger proteins, it has been suggested (Goldberg, 1969), that distinct regions of the polypeptide chain are able to fold independently, before specifically interacting with each other. One way of checking this model is to isolate the polypeptide fragment corresponding to such a region and to test whether this fragment by itself is able to fold into the conformation it adopts in the native protein. Such a study has been undertaken to investigate the structure and folding of the β_2 subunit of *E. coli* tryptophan synthetase. In a previous report (Högberg-Raibaud and Goldberg, 1977), it has been shown that the protomer of this enzyme can be hydrolyzed by trypsin to give two polypeptide fragments F_1 and F_2 of molecular weights 29 000 and 12 000, respectively. It has been shown that the "nicked β_2 protein", like the intact native enzyme, is a dimer. Though inactive, it is able to bind the coenzyme (pyridoxal-P) and the substrates (indole and L-serine) of the native enzyme. In the presence of L-serine, it also exhibits the fluorescence properties of an intermediate, the "aqua"-complex, in the catalytic reaction performed by β_2 (Goldberg et al., 1968). Therefore, the conformations of F_1 and F_2 in the nicked and intact protein appear to be very similar.

In an attempt to establish whether fragments F_1 and F_2 correspond to independently folding regions of the β chain of tryptophan synthetase, these fragments had to be isolated and their folding properties investigated. In the present paper, it is shown that F_1 and F_2 can be separated under denaturing conditions and that native nicked β_2 can be recovered upon mixing F_1 and F_2. Furthermore, it is shown that the isolated fragments are able to spontaneously refold, upon removal of the denaturant, into globular structures which will be characterized by their hydrodynamic and optical properties.

Materials and Methods

Purification of the β_2 Subunit. Bacterial Strain and Growth Conditions. The enzyme was isolated from *E. coli* strain Δtrp ED102/F′ trp ED102, which produces high amounts of the $\alpha_2\beta_2$ protein. The bacteria were grown on Vogel-Bonner minimal medium (Vogel and Bonner, 1956), supplemented with 0.05% acid hydrolyzed casein, 0.4% glucose, and 8 μg/mL of L-tryptophan, and the growth took place in a Fermatron (New Brunswick Scientific Co.) fermentor at 37 °C under vigorous aeration, during 20 h. The growth medium was then neutralized, and the cells were harvested by centrifugation in a Sharples centrifuge and frozen.

Purification Conditions. The standard buffers used were 0.1 M potassium phosphate buffer, pH 7.8, containing 10^{-2} M β-mercaptoethanol and 2×10^{-3} M EDTA[1] (buffer A), and buffer A supplemented with 10^{-4} M pyridoxal-P and 5×10^{-4} M phenylmethanesulfonyl fluoride (buffer B). The purification was, if not otherwise indicated, carried out in the dark and at 4 °C. All centrifugations were made at 25 000*g* in a Sorval RC2-B centrifuge. The purification consists of the following steps.

Step 1: Preparation of Crude Extract. Twenty grams of bacteria (wet weight) was suspended in 40 mL of buffer B. The cells were disrupted by sonication in a Branson sonifier and the cell debris were eliminated by centrifugation for 30 min. The supernatant was recentrifuged once, while the pellet was washed with 10 mL of the same buffer. The pooled supernatants were then diluted to 100 mL with distilled water, con-

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[1] Abbreviations used: EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

taining 10^{-2} M β-mercaptoethanol, and 10^{-4} M pyridoxal-P.

Step 2: Treatment with Protamine Sulfate. The pH of the diluted crude extract was lowered to 6.0 with 1 M acetic acid. The solution was kept under stirring at 14 °C and 14 mL of protamine sulfate (from a 2% neutralized solution in water) was then added dropwise. After 5 min the suspension was centrifuged for 15 min and the pH of the supernatant was immediately brought to 7.8 with 1 M KOH.

Step 3: Differential Ammonium Sulfate Precipitation of the $\alpha_2\beta_2$ Complex. To the neutralized supernatant from step 2, solid ammonium sulfate was added under agitation to 35% saturation (Green and Hughes, 1955), and the pH was adjusted to 7.5 with 1 M KOH. After 30 min the precipitate was eliminated by centrifugation for 15 min. The supernatant was supplemented with ammonium sulfate until 65% saturation, adjusted to pH 7.5, and was then left for 30 min. The suspension was centrifuged as before; the supernatant was discarded, while the active precipitate was dissolved in 10 mL of buffer B. It was then dialyzed against the same buffer, first for 3 h at room temperature against 500 mL and then overnight at 4 °C against 1 L.

Step 4: Elimination of the α Subunit by Heat Treatment. The dialyzed enzyme was heated in a 50-mL glass tube in a water bath at 63 °C during 10 min. The tube was then immediately cooled, and precipitated protein was removed by centrifugation for 15 min. The supernatant was collected and the pellet washed once with 8 mL of buffer A. The two supernatants were then pooled.

Step 5: Resolution of the β_2 Subunit. As previously reported (Adachi and Miles, 1974), the β_2 subunit crystallizes in the apo form and has therefore to be resolved before crystallization. For this purpose the supernatant from step 4 was incubated for 20 min at 37 °C with 10^{-2} M hydroxylammonium chloride (from a 1 M neutralized stock solution). A 90% saturated solution of ammonium sulfate was then added dropwise until 40% saturation. After 30 min the precipitate was collected by centrifugation for 15 min. The pellet was dissolved in 2.5 mL of buffer A and the treatment with hydroxylammonium chloride was repeated. The enzyme was then dialyzed overnight against 500 mL of ammonium sulfate (5% saturation) in buffer A.

Step 6: Crystallization. The crystallization was initiated by inoculation with about 0.4 mg of crystallized β_2 from an earlier preparation. The concentration of ammonium sulfate was then raised by 1.25% saturation each day up to 10% by adding small aliquots of the 90% stock solution in buffer A. The suspension was kept for 1 more day and was then centrifuged. The supernatant was discarded and the crystalline precipitate was washed once with ammonium sulfate (37.5% saturation) in buffer A. The crystals were finally suspended in the same buffer and kept at 4 °C.

Reactivation of the β_2 Subunit. The crystalline suspension, kept at 4 °C, slowly looses its activity, but could be fully reactivated in the following way. The required amount of crystals was centrifuged, dissolved in 0.1 M potassium phosphate buffer, pH 8.0, containing 2% ammonium sulfate, 2×10^{-3} M EDTA, and 2.5×10^{-2} M dithiothreitol, and incubated 1 h at 50 °C. It was then transferred into the desired buffer by filtration on a small Sephadex G-25 column.

Preparation and Isolation of the Tryptic Fragments. The proteolysis of the β_2 subunit was performed in 0.05 M Tris-HCl buffer, pH 7.8, containing 2×10^{-3} M EDTA, 5×10^{-3} M β-mercaptoethanol, and 5×10^{-5} M pyridoxal-P (buffer C), at 25 °C and in the dark. If not otherwise indicated, the concentration of β_2 was 20 mg/mL and the trypsin (*N*-tosyl-L-phenylalanine chloromethyl ketone treated bovine pancreatic trypsin from Worthington) was added to a final concentration of 5 μg/mL. The proteolysis was stopped, when the residual activity reached 1-2%, by passing the protein through a small column of soybean trypsin inhibitor, immobilized on Sepharose (Cuatrecasas et al., 1968).

The nicked protein was then dialyzed for 5 h at 4 °C in the dark against 6 M urea in 0.1 M potassium phosphate buffer, pH 7.8, containing 0.1 M β-mercaptoethanol and 2×10^{-3} M EDTA. After dialysis the tryptic fragments were separated on a column of Sephadex G-100, equilibrated with the same urea solution. The fractions of each peak were pooled, and each pool was dialyzed separately at 4 °C in the dark, first for 6 h against 0.1 M potassium phosphate buffer, pH 7.8, containing 0.1 M β-mercaptoethanol, 10^{-5} M pyridoxal-P, and 2×10^{-3} M EDTA and then overnight against the same buffer, except for the β-mercaptoethanol which was 2×10^{-3} M ("renaturation buffer").

Activity and Protein Assays. The activity of the β_2 subunit was measured in the indole to tryptophan reaction in the presence of an excess of α subunit to activate the β_2 (Hatanaka et al., 1962). The spectrophotometric assay of Faeder and Hammes (1970) was usually performed (at 25 °C), but the conventional radioactive assay (Miles, 1970) was also used to measure the specific activity of the purified enzyme.

Protein concentrations were either estimated by the procedure of Lowry et al. (1951), using bovine serum albumin (Fraction V powder, Sigma) as a reference standard, or spectrophotometrically at 280 nm. The specific absorbance used for the holo-β_2 subunit was $E_{280}^{1\%} = 6.5$ (Miles, 1970) and for each fragment it was determined from the absorbance and the amino acid analyses of the samples.

Amino Acid Analysis. Quantitative amino acid analyses were performed in a Jeol JLC-5AH analyzer after hydrolysis with 6 N HCl at 110 °C in evacuated sealed tubes during 24 and 48 h. Each sample contained 25 μg of protein. The values for serine and threonine were corrected for the destruction during hydrolysis by extrapolation to zero time.

Gel Electrophoresis. Disc electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate was performed according to Shapiro et al. (1967).

Analytical Centrifugations. The centrifugations were carried out in a Centriscan 75 (M.S.E., England) analytical ultracentrifuge equipped with a monochromator, using the ultraviolet absorption scanning system at 280 nm. Molecular weights were measured by sedimentation equilibrium at high speed according to Yphantis (1964). The partial specific volume used for calculations was 0.738 mL/g (Hathaway, 1972).

Fluorescence Measurements. Fluorescence measurements were performed at room temperature by use of a double monochromator CGA (DC 3000/1) spectrofluorimeter equipped with a Servotrace PE (Sefram, Paris) recorder. The optical density of the sample throughout the excitation range was below 0.1 and the internal filter effect could thus be neglected. In the spectra reported, the fluorescence of the solvent has been subtracted.

Circular Dichroism Spectra. The circular dichroism spectra were recorded on the dichrograph designed, constructed, and calibrated by Drs. J. Brahms and S. Brahms (manuscript in preparation). The samples were analyzed at room temperature in 1-mm thick cells to keep the total absorbance of the samples below 0.2 throughout the spectrum. The scanning speed was 2 nm/min. The amount of secondary structure structure was estimated from the CD spectra according to Greenfield and Fasman (1969).

TABLE I: Purification of the β_2 Subunit.[a]

Purification step	Volume (mL)	Enzyme act. (EU)	Protein content (mg)	Specific act. (EU/mg)	Yield in act. (%)
1	100	62 700	2200	29	100
2	110	72 300	1800	40	115[c]
3	22	67 000	1000	67	107
4	26	77 100	300	260	123[c]
5	3	68 100	110	600	109
6	2	50 200	50	1000[b] (at 37 °C: 3300)	80

[a] The purification was started from 20 g (wet weight) of bacteria. The assay for enzymatic activity was performed at 25 °C as described in Materials and Methods. The α subunit was, however, omitted from the assay mixture for the first three steps since, before heating at 63 °C, the β_2 protein is saturated with α. [b] At 37 °C; 3300. [c] Though variable in extent from one preparation to another, a significant reactivation has been reproducibly observed during the protamine sulfate precipitation and the heat step.

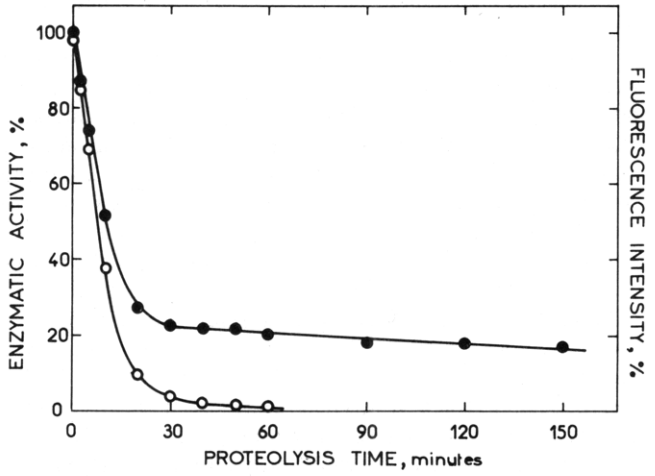

FIGURE 1: Kinetics of proteolysis. A solution (8 mg/mL) of the β_2 subunit in buffer C was treated with 4 μg/mL of trypsin at 25 °C. At the times indicated in the abscissa, 10-μL aliquots were removed and added to 1 mL of soybean trypsin inhibitor (10 μg/mL) in 0.1 M potassium phosphate buffer, pH 7.8, containing 10^{-5} M pyridoxal-P, 2×10^{-3} M β-mercaptoethanol, and 2×10^{-3} M EDTA. Each aliquot was then assayed for activity and the fluorescence intensity was measured at 510 nm (λ_{ex} = 440 nm) after adding 0.1 M L-serine. (O—O) Enzymatic activity; (●—●) fluorescence intensity.

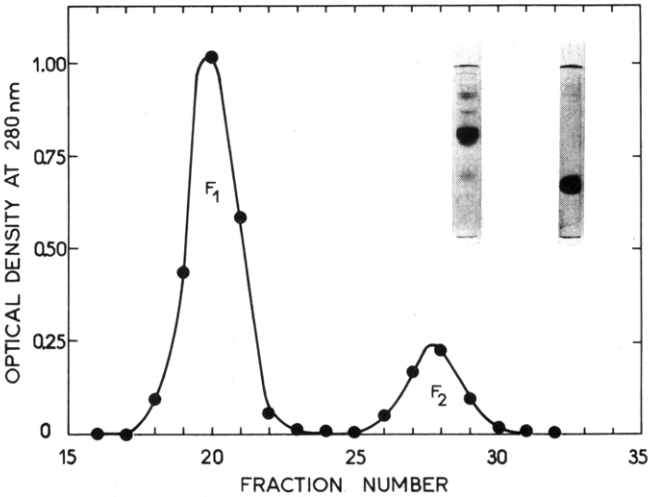

FIGURE 2: Separation of the fragments. A sample of nicked protein (11 mg in 1 mL), previously dialyzed against 6 M urea, was applied to a 1.7 × 52 cm column of Sephadex G-100 superfine, equilibrated in the same urea solution. (See Materials and Methods.) The column was run at 4 °C, and the flow rate was 6 mL/h. Fractions of 2 mL were collected and the optical density of each fraction was measured at 280 nm. The inset shows sodium dodecyl sulfate gels of the pooled fractions of each peak.

Results

Purification of the β_2 Protein. Previously reported methods for purifying the β_2 subunit of tryptophan synthetase appeared to be either time consuming or poorly reproducible. Therefore, a new very simple, rapid, and reproducible method has been devised, which yields pure crystallized protein. It is based on the large difference between the ammonium sulfate precipitation curves of the $\alpha_2\beta_2$ complex and of the β_2 subunit (Adachi et al., 1974; Adachi and Miles, 1974) and on the difference in thermal stability of the α and β_2 subunits (Miles, 1970).

The results of a typical purification are described in Table I.

The protein obtained appeared homogeneous by electrophoresis on polyacrylamide and its specific activity is characteristic of pure enzyme (Adachi and Miles, 1974).

Preparation of the F_1 and F_2 Fragments. The kinetics of proteolysis by trypsin of the β_2 protein has been followed by monitoring both the enzymatic activity and the fluorescence intensity of the aqua complex. Figure 1 shows that the fluorescence intensity first undergoes a rapid decrease down to 20% of its initial value and that the rate of this decrease is equal to the rate of the inactivation. Since it had been shown previously (Högberg-Raibaud and Goldberg, 1977) that the loss of activity strictly corresponds to the appearance of the nicked protein, it can be concluded that the rate of the formation of the nicked protein and the rate of the initial decrease of fluorescence intensity are identical. It also can be concluded that the fluorescence intensity exhibited by the nicked protein is 20% of that of the intact β_2 protein. The further very slow decrease of the fluorescence intensity upon proteolysis, as seen in Figure 1, is likely to be due to a slow degradation of the nicked β_2 protein by trypsin. In order to avoid this degradation, the trypsin treatment was, from then on, stopped when the inactivation of β_2 had reached 98 to 99%.

To achieve the separation of the fragments, the nicked protein was denatured with 6 M urea and filtered through a Sephadex G-100 column, equilibrated with the same urea solution. The elution pattern, given in Figure 2, shows two protein peaks. Electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate shows (inset to Figure 2) that each peak contains a pure fragment, the migration of which corresponds to that previously described for fragments F_1 and F_2, respectively (Högberg-Raibaud and Goldberg, 1977). It thus appears that these fragments can be dissociated from each other in 6 M urea and isolated by gel filtration. The fractions of each peak were pooled and urea was removed by dialysis as described in Materials and Methods; both F_1 and

TABLE II: Amino Acid Analysis of the Fragments.[a]

Amino acid	Mol per mol of F_1 of 29 000 MW	Mol per mol of F_2 of 12 000 MW	Mol per mol of β_2 of 45 000 MW
Lys	15	6-7	23
His	10	5-6	17
Arg	15	4	22
Asp	18	11	34
Thr	17	5	23-24
Ser	11	8-9	20
Glu	32-33	13-14	50
Pro	15	5-6	20-21
Gly	34	9	45
Ala	28-29	10	45-46
Val	11-12	4	21
Met	7-8	3-4	13
Ile	12	6-7	23-24
Leu	26-27	11	40
Tyr	7-8	3	11
Phe	8-9	3	13
Trp	1	0	1-2

[a] For each polypeptide chain, three independent samples were analyzed for each hydrolysis time. The table shows the mean values calculated from these samples. The tryptophan was determined spectrophotometrically by the method of Edelhoch (1967).

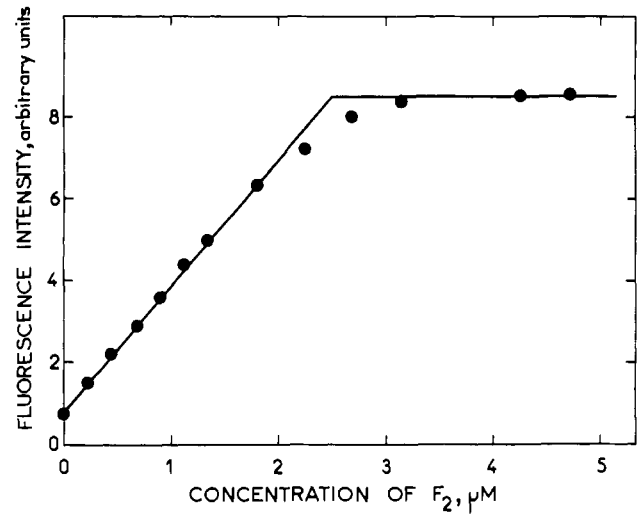

FIGURE 3: Titration of F_1 with F_2. The fragments were prepared as described in Materials and Methods. F_1 was diluted to a final concentration of 3.5 μM into the "renaturation buffer", containing 0.1 M L-serine. To a 0.9-mL sample of this fragment, small aliquots of F_2 were added, and the fluorescence intensity was measured at 510 nm (λ_{ex} = 440 nm). The concentration of each fragment is expressed as the molarity of polypeptide chain.

F_2 remained soluble during this treatment. These dialyzed preparations will hereafter be called the "isolated fragments".

The amino acid composition of each fragment was then determined and is shown in Table II.

Finally, the specific extinction coefficients of the isolated fragments were measured. The values obtained were:

$$\epsilon_{280}^{mg/mL} = 0.67 \quad \text{for } F_1$$

and

$$\epsilon_{280}^{mg/mL} = 0.45 \quad \text{for } F_2$$

Reconstitution of the Nicked Protein from the Isolated Fragments. The fluorescence of F_1 and F_2 in the presence of pyridoxal-P and L-serine was investigated to establish whether one of the isolated fragments alone would give rise to the aqua complex. F_2 gave no fluorescence and F_1 produced only a small fluorescence signal. However, when F_1 and F_2 were mixed, the intense fluorescence of the aqua complex reappeared. Figure 3 shows a titration experiment where, to a constant amount of F_1, aliquots of F_2 were added. The fluorescence intensity reached a plateau when a stoichiometric amount of F_2 was added to F_1. Moreover, the specific fluorescence of the stoichiometric mixture is very close (80–90%) to that of the initial nicked β_2 protein.

The fluorescence properties of the reconstituted protein were analyzed in detail and compared with those of the original nicked protein, previously reported by Högberg-Raibaud and Goldberg (1977). First, the excitation spectra (λ_{em} = 510 nm) were identical for the two proteins in the presence of pyridoxal-P, with the same maximum at 412 nm, characteristic of a Schiff base formed between the pyridoxal-P and the protein. Upon addition of L-serine, the same red shift of the excitation maximum and the same increase in fluorescence intensity were observed.

Furthermore, the dissociation constant of the reconstituted nicked protein for L-serine was measured as for the original nicked protein and found to be K_D = 13 ± 5 mM; this value is within experimental error the same as that reported for the nicked protein (K_D = 20 ± 5 mM). Similarly, the effect of indole on the fluorescence of the stoichiometric mixture was investigated. As for the nicked protein, it was observed that indole quenches the fluorescence of the aqua complex elicited by the reconstituted protein mixture. The dissociation constant for indole was measured and found to be K_D = 4 ± 2 mM, which is within experimental error identical with that measured in the same way for the nicked protein (K_D = 6 ± 2 mM).

The identity of their fluorescence spectra in the absence and presence of L-serine and of their dissociation constants for L-serine and indole show that, by these functional criteria at least, the reconstituted protein and the initial nicked β_2 protein are very similar.

It therefore can be concluded that, after having been denatured and separated in 6 M urea, the F_1 and F_2 fragments can be renatured to yield back upon mixing the functional properties (i.e., binding sites for pyridoxal-P, L-serine, and indole) of the nicked protein. That this result is not due to a failure of urea to denature the fragments is shown by the fact that the same renaturation was observed after submitting the fragments to 6 M guanidine hydrochloride, which is known to be a much stronger denaturing agent.

Physical Characterization of the Reconstituted Nicked Protein. The structural properties of the stoichiometric mixture of F_1 and F_2 have also been investigated to ascertain that this protein is identical with the original nicked protein.

First, its sedimentation coefficient has been measured. The value obtained, $s_{20,w}$ = 4.5 S, is not significantly different from that reported for the nicked protein (4.6 S). Hence, it can be concluded that the reconstituted nicked protein has the same quaternary structure as the original nicked β_2 (i.e., two F_1 and two F_2 fragments, tightly associated).

Next, the fluorescence of the aromatic residues of the nicked protein and of the reconstituted protein have been examined. Figure 4 shows that the two emission spectra are within experimental errors identical, with a maximum at 327 nm in buffer and a maximum at 355 nm in a denaturing solvent. This result shows that in the reconstituted protein the tryptophan residue responsible for the fluorescence is in the same environment as in the nicked protein, and moreover that this tryptophan is not accessible to the solvent (Pajot, 1976).

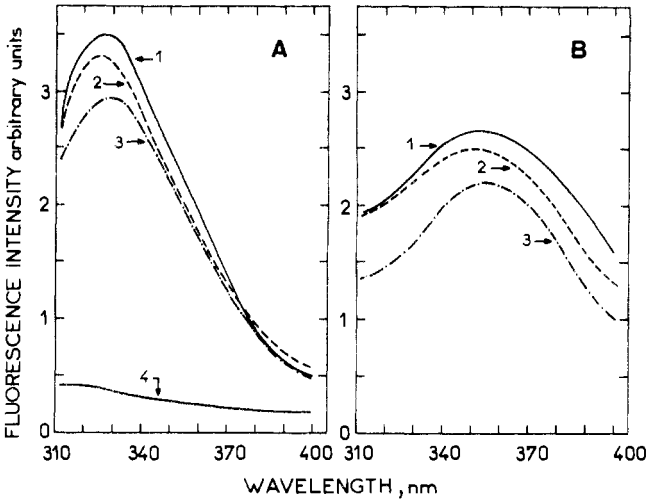

FIGURE 4: Fluorescence emission spectra of the aromatic residues. The nicked protein and the isolated fragments were obtained as indicated in Materials and Methods. Each sample was diluted to a final concentration of 5 μM (calculated per monomer) either into 0.1 M potassium phosphate buffer, pH 7.8, containing 5×10^{-2} M β-mercaptoethanol and 2×10^{-3} M EDTA (A), or into the same buffer supplemented with 6 M guanidine hydrochloride (B). The spectra were recorded 0.5 h after the dilution. (1) Nicked β_2; (2) a stoichiometric mixture of each fragment; (3) the F_1 fragment; and (4) the F_2 fragment. The excitation wavelength was 280 nm.

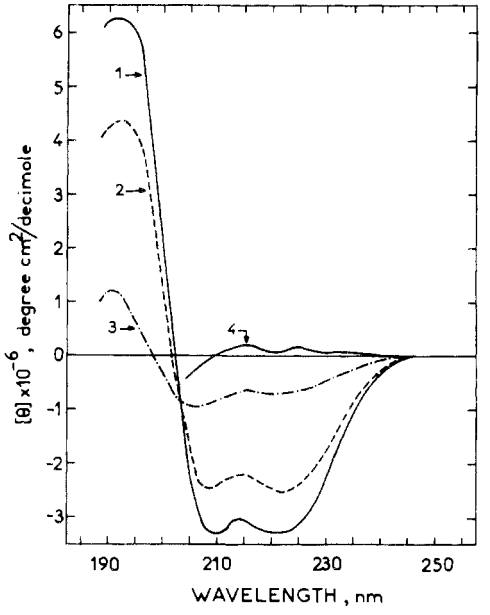

FIGURE 5: Circular dichroism spectra of the fragments. The nicked β_2 and the isolated fragments were prepared as described in Materials and Methods. Before recording the spectra, each sample was filtered on a Sephadex G-25 column, equilibrated with 0.02 M potassium phosphate buffer, pH 7.8. The ellipticity of each sample is expressed per decimole of polypeptide chain. (1) Nicked β_2 or a stoichiometric mixture of the fragments (identical spectra were obtained); (2) the F_1 fragment; (3) the F_2 fragment; and (4) the calculated difference spectrum between the sum of the spectra of each fragment and the spectrum recorded for the stoichiometric mixture.

Finally, the secondary structures of the reconstituted protein and of the original nicked protein have been investigated by recording their circular dichroism spectra in the far UV. Figure 5 shows that the spectra of the two proteins are indentical. The amount of secondary structure which can be estimated from these spectra corresponds to about 15% α helix and 30–40% β structure.

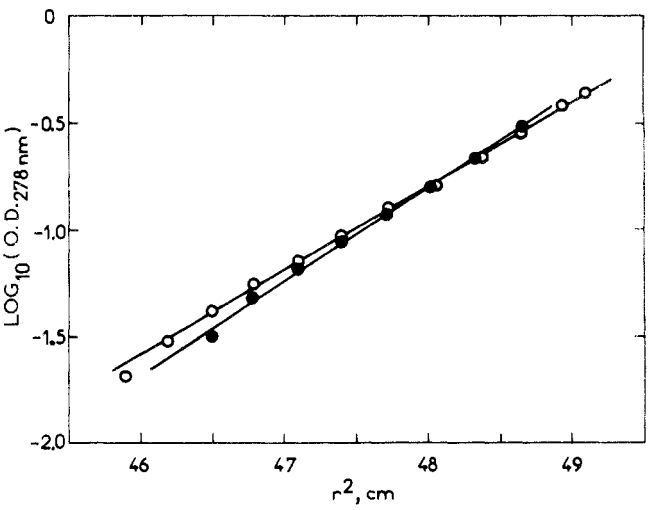

FIGURE 6: Sedimentation equilibrium of the renatured fragments. After dialysis against the "renaturation buffer", each fragment was diluted to 0.5 mg/mL and spun at 25 000 rpm and 8 °C. Equilibrium was reached after 18 h. (●—●) The F_1 fragment; (○—○) the F_2 fragment.

Thus, in addition to having regained the functional properties of the nicked protein, the reconstituted protein has also recovered all the physical properties so far examined of the original nicked protein.

It therefore can be concluded that, in the reconstituted protein, the fragments F_1 and F_2 have been able to regain the conformation which they originally had in the nicked protein. However, in order to show that these fragments correspond to independently folding regions of the intact polypeptide chain, it had to be demonstrated that they could reach their native secondary-tertiary conformation independently of each other, i.e., before being mixed together. For this reason, a study of the isolated fragments has been undertaken.

Physical Characterization of the Isolated Fragments. As mentioned above, neither F_1 nor F_2 alone exhibited the intense fluorescence of the aqua complex which allowed a straightforward study of the ligand binding properties of the nicked protein. Therefore the conformation of the isolated fragments was investigated by a physical approach.

First, their sedimentation coefficients were measured by analytical centrifugation. The values found were $s_{20,w} = 2.5$ S for F_1 and $s_{20,w} = 2.1$ S for F_2. Next, their molecular weights were determined by sedimentation equilibrium. Figure 6 shows that both preparations were homogeneous in molecular weight. The values calculated from the slopes of the lines in Figure 6 were 27 000 daltons for F_1 and 24 000 daltons for F_2. While F_1 is clearly a monomer, the isolated F_2 fragment thus appears to be tightly associated into dimers. Moreover, from the values found for the sedimentation coefficient and molecular weight of each preparation, the values of $f/f_0 = 1.3$ and $f/f_0 = 1.4$ are obtained for F_1 and F_2, respectively. Thus, from their hydrodynamic properties both proteins appear as compact globular structures (Oncley, 1941).

The fluorescence of the aromatic residues of the isolated fragments has also been investigated. F_2 has no tryptophan (Table II) and exhibits only a weak fluorescence, due to its tyrosine residues. F_1, which contains one tryptophan, shows a fluorescence with a maximum emission wavelength of 327 nm, characteristic of a tryptophan in an hydrophobic environment (Figure 4A). This clearly indicates that the isolated F_1 fragment is folded in such a way as to bury its tryptophan residue away from the solvent. The comparison of the emission spectrum of the nicked protein with the spectrum of F_1 (Figure

4) shows that this fragment produces the same fluorescence (maximum emission wavelength and intensity), when either isolated or within the nicked protein. It thus can be concluded that the association of F_1 and F_2 does not produce a change in the conformation of the F_1 fragment, sufficient to affect its fluorescence properties.

Finally, the circular dichroism in the far UV of the isolated fragments has been investigated. From the spectra shown in Figure 5, the content of α helix and β structure in each isolated fragment has been estimated, indicating about 15% α helix for both fragments, 30–40% β structure for F_1, and 15–25% β structure for F_2. These results show that both fragments contain important amounts of secondary structure.

Moreover we computed the difference spectrum between the circular dichroism of the stoichiometric mixture of F_1 and F_2, on one hand, and the sum of the circular dichroisms of the isolated fragments, on the other hand. That this difference does not deviate from zero throughout the spectrum (Figure 5) clearly indicates that the reassociation of the isolated fragments into the nicked protein does not produce a significant change in their secondary structures.

From the set of results reported above, it can be concluded that the isolated F_1 and F_2 fragments are able to refold independently of each other into compact, monodisperse, globular conformations containing the same secondary structures which exist in the corresponding fragments of the nicked protein.

Discussion

That the β_2 subunit of *Escherichia coli* tryptophan synthetase can be renatured after treatment with 6 M urea had already been reported by Hathaway et al. (1969). In the present paper, the renaturation of the nicked protein, obtained by a limited proteolytic cleavage of β_2, has been studied. It is shown that by treatment with 6 M urea or 6 M guanidine hydrochloride the polypeptide chains which build up the nicked protein (i.e., two F_1 and two F_2 fragments) are dissociated and can be readily isolated in the denatured state by gel filtration. By removing the denaturing agent and mixing back the F_1 and F_2 fragments in stoichiometric amounts, one obtains a protein which, by its physical properties (sedimentation coefficient, tryptophan fluorescence, circular dichroism) and functional properties (binding of the substrates and cofactor, formation of the aqua complex) is indistinguishable from the native nicked β_2 protein. It therefore can be concluded that, in spite of the fact that the β chain has been cleaved in two fragments, the nicked protein is able to spontaneously refold into its original conformation. This finding by itself eliminates a model according to which the folding process must start at the N-terminal side and propagate along the polypeptide chain. It also shows that the residues which have been lost during the proteolysis (corresponding to 4000 daltons per β chain) are not required for the refolding of the nicked protein. Thus, in the folding of the intact β chain, these residues are likely to interact with the regions corresponding to the F_1 and F_2 fragments only after these regions have achieved the conformation of the nicked protein.

It has also been shown that F_1 alone as well as F_2 alone are able to spontaneously refold into conformations which have been characterized as follows. They appear as compact globular structures having approximately the same α helix and β structure content as in the nicked protein. Hence, the secondary structure and overall shape of the isolated fragments seem to be the same as in the corresponding fragments within the nicked protein. Moreover, each fragment contains an additional probe which allows comparison of its conformation in the isolated or completed state. For F_1, the fluorescence properties of the tryptophanyl residue show that this residue is buried in an hydrophobic environment. The fact that the fluorescence emission spectra are identical in the isolated fragment and in the nicked protein strongly suggests that the tertiary structure which creates this hydrophobic environment is similar in the isolated F_1 and in the nicked protein.

The isolated F_2 fragment has regained upon folding at least one specific property of the nicked protein and of the β_2 subunit: it is a dimer. It is therefore tempting to conclude that in the nicked protein and in the native β_2 subunit the association areas between the protomers are carried by the region corresponding to the F_2 fragment. That the isolated F_2 fragments also carry these stereospecific association areas then seems to be strong evidence of a close fit between the conformations of F_2 within the nicked protein and the isolated fragment.

The results discussed above clearly show that both isolated fragments are able to fold into conformations which approximate quite well the structure of the corresponding fragment within the nicked protein and, as discussed earlier (Högberg-Raibaud and Goldberg, 1977), of the corresponding region within the intact native enzyme. These fragments thus correspond to independently folding regions, previously designated as "globules" (Goldberg, 1969), of the β chain. By analogy with the complementation between the F_1 and F_2 fragments which yields "functional" nicked β_2, it is most probable that the folding of the intact β chain proceeds through the folding of these globules, followed by their intrachain association to yield the native protomer. The isolated F_1 and F_2 fragments therefore appear as intermediate structures in the folding of the β_2 subunit of *Escherichia coli* tryptophan synthetase, and it can be predicted that they correspond to distinct domains of the native enzyme.

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Characterization of the Purified Membrane Attachment (δ) Subunit of the Proton Translocating Adenosine Triphosphatase from *Escherichia coli*†

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ABSTRACT: Some of the physical and functional characteristics of the purified δ subunit obtained from the proton-translocating ATPase of *Escherichia coli* (ECF_1) have been examined. The subunit has a molecular weight of about 18 500 as measured by sodium dodecyl sulfate electrophoresis and by sedimentation equilibrium either with or without 6 M guanidine hydrochloride. δ therefore exists as a monomer and the apparent high molecular weight of about 33 000 obtained from molecular sieve chromatography suggests that the protein is a rather elongated molecule with a calculated f/f_0 of 1.4. Circular dichroism spectra indicate that δ has a high degree of secondary structure with an α-helix content of about 55-70%. The amino acid composition was determined. δ attaches δ-deficient ECF_1 to inverted membrane vesicles depleted of ECF_1 and restores oxidative phosphorylation. About 1 part δ by weight in the presence of excess ECF_1 lacking δ reconstituted depleted membranes to the same extent as 20 parts of completely reconstitutive ECF_1. This indicates that only one δ of mol wt 18 500 is needed per functional ECF_1 molecule of mol wt 370 000. δ associates rapidly with δ-deficient enzyme to yield a stable five-subunit complex which was separated from excess δ by molecular sieve chromatography and was fully active in reconstituting depleted membranes. δ has no effect on activities in depleted or partially reconstituted membrane vesicles and binds only poorly, if at all, to these membranes. It appears then that reconstitution with δ is an ordered process in which δ first combines with δ-deficient ECF_1 to yield a complex which then binds to membranes.

The Mg^{2+}-dependent ATPase located in the cytoplasmic membrane of *Escherichia coli* is the catalytic unit which reversibly transduces the energy between an electrochemical gradient and ATP.[1] The complete enzyme is composed of several nonidentical polypeptides which belong to one or the other of two morphologically and biochemically distinct portions. The portion designated F_0, which is intrinsic to the membrane, acts as a proton ionophore and a receptor for the other portion referred to as F_1. The F_1 portion, which is peripheral to the membrane, contains the catalytic site as well as the high affinity binding sites for nucleotides. F_1 dissociates readily from the F_0 in the membrane as a water-soluble protein. The catalytic and structural features of the F_0-F_1 complex from *E. coli* are remarkably similar to those found for the proton-pump ATPases of mitochondria, chloroplasts, and other bacteria (see reviews by Pedersen, 1975; Racker, 1976; Harold, 1977).

The purified F_1 portion of the ATPase contains five different polypeptides (α, β, γ, δ, and ϵ) which, in the case of *E. coli*, range in size from about 60 000 for the α subunit (Bragg and Hou, 1972) to 16 000 for ϵ (Smith and Sternweis, 1977). The authenticity of each of these polypeptides as an F_1 subunit is suggested by their occurrence in all preparations of F_1 capable of reconstituting a variety of energy transducing reactions catalyzed by the complete F_0-F_1 complexes. However, definitive evidence requires one to show that each polypeptide is indeed a functional unit of F_1. This is especially critical when considering the two smaller F_1 subunits (δ and ϵ) since they represent a relatively minor amount of the total F_1 molecule, which is comprised mostly of α and β chains. ϵ is believed to

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[1] Abbreviations used are: ATP, adenosine triphosphate; F_0, the portion of the proton-translocating ATPase which is an integral component of the membrane; F_1, the portion of the proton-translocating ATPase which is peripheral to the membrane; α, β, γ, δ, ϵ, separate polypeptides of F_1 molecules in order of decreasing size; CF_1, F_1 from chloroplasts; ECF_1, F_1 from *E. coli;* 4-subunit F_1, F_1 containing only the α, β, γ, and ϵ subunits; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; NADH, reduced nicotinamide adenine dinucleotide; EDTA, (ethylenedinitrilo)tetraacetic acid; DTT, dithiothreitol; BSA, bovine serum albumin; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.