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Biochemistry

Preparation and characterization of a modified form of β_2 subunit of *Escherichia coli* tryptophan synthetase suitable for investigating protein folding

(limited proteolysis/domains/complementation)

ANNA HÖGBERG-RAIBAUD AND MICHEL E. GOLDBERG

Unité de Biochimie Cellulaire, Institut Pasteur, 75724 Paris, Cedex 15, France

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ABSTRACT **Globular proteins often appear to consist of distinct compact "domains," and the assumption is frequently implicitly made that these domains correspond to intermediate structures in the folding process. If this assumption is correct, the polypeptide fragment that builds up a domain should be able to spontaneously fold into its native conformation even when isolated. In an attempt to isolate and study such a fragment, the β_2 subunit of tryptophan synthetase [tryptophan synthase, L-serine hydro-lyase (adding indoleglycerol-phosphate), EC 4.2.1.20] has been subjected to controlled proteolysis. The resulting protein is shown to be a dimer, the protomer of which contains two nonoverlapping polypeptide chains of molecular weights 12,000 and 29,000. Though inactive, the nicked protein is shown to be in a conformation that closely resembles that of the original enzyme, since it still can form an enzyme-bound intermediate of the catalytic reactions. The fluorescence of this intermediate is used to characterize the binding sites for the cofactor (pyridoxal-*P*) and substrates, which are shown to exist on the nicked protein. The possibility is discussed of using the fragments isolated from the nicked protein to study individual steps of the enzymatic reaction, intracistronic complementation, and the folding process in the normal β_2 subunit.**

Since Phillips first inferred that the "compact globular units" he observed in the structure of lysozyme might reflect some features of the protein folding (1), more emphasis has been put on the kinetic, rather than mere energetic, properties of the formation of native protein structures. Using the same reasoning as Phillips, Wetlaufer (2) argued that the observation of distinct compact regions, or "domains," in most globular proteins favors a sequential model whereby the domains would fold independently before interacting with each other to form the native structure. In turn, Levitt and Chothia (3) proposed that "locally ordered regions, which are referred to here as folding units, associate to form the whole protein molecule, or in the case of some of the larger proteins, to form domains."

However attractive these models may appear, they are still quite hypothetical since they originate essentially from considerations on the conformation of native proteins, i.e., the *result* of the folding and not the *process* of folding. For instance, no convincing direct evidence is available to support the assumption that a folded domain corresponds to an intermediate structure in the folding of the whole polypeptide chain.

A possible approach to a test of this assumption is to isolate, from the native protein, the fragment of the polypeptide chain that corresponds to a domain and investigate its folding and structural properties. If the domain is indeed closely related to a structural intermediate in the folding process, the corresponding isolated peptide should be able to spontaneously fold into a conformation that closely resembles that of the domain in the native protein.

Such a study has been undertaken with the β_2 subunit of *Escherichia coli* tryptophan synthetase [tryptophan synthase L-serine, hydro-lyase (adding indoleglycerol-phosphate), EC 4.2.1.20]. This protein was chosen for the following reasons: first, it can be easily renatured after denaturation (4), and this is essential for studying the folding properties of the domains. Second, it is able to interact with several specific ligands (the α subunit; the coenzyme, pyridoxal-*P*; and the two natural substrates, L-serine and indole) whose binding can be easily monitored and used as a probe of the protein conformation. In the present paper, we describe some physical and functional properties of a modified form of the β_2 protein, obtained by a mild proteolytic treatment aimed at nicking the polypeptide chain in between two domains. The possibility of using this modified protein to isolate a domain and prove that it is an intermediate structure in the folding of the β_2 protein is discussed.

MATERIALS AND METHODS

Enzymes. Crude extracts of the α subunit were obtained as described by Henning *et al.* (5). The β_2 subunit was prepared by a combination of two previously reported methods (6, 7) and had a specific activity of 2700–3000 units/mg in the indole to tryptophan reaction. Apo-β_2 was obtained by incubation of the holoenzyme (15 min at 37°) with 2 mM hydroxylammonium chloride.

Activity and Protein Assays. The synthesis of L-tryptophan from indole and L-serine was followed either by the conventional radioactive assay (8) during the purification of β_2 or by the kinetic assay of Faeder and Hammes (9). Unless otherwise stated, these assays were performed in the presence of an excess of α subunit to activate β_2 (10). The assay of the L-serine deamination reaction of β_2 was that of Crawford and Ito (11).

Indoleglycerol-phosphate hydrolysis was measured spectrophotometrically as described by Creighton and Yanofsky (12), with pure α subunit and indoleglycerol-phosphate, which were generous gifts from Dr. K. Kirschner.

The activity of trypsin on tosyl-L-arginine methylester was measured as indicated earlier (13).

Protein concentrations were estimated by the procedure of Lowry *et al.* (14), with bovine serum albumin (Fraction V powder, Sigma) as a reference standard.

Conditions of Proteolysis. Bovine pancreatic trypsin (315 units/mg) that had been treated with *N*-tosyl-L-phenylalanine chloromethyl ketone was obtained from Worthington. A stock solution containing 5 mg/ml of trypsin in 1 mM HCl was kept frozen until needed. Proteolysis was performed in 50 mM Tris·HCl, pH 7.8 (at 20°) containing 2 mM EDTA, 5 mM 2-mercaptoethanol, and 50 μM pyridoxal-*P* (buffer A) at room temperature and in the dark to protect the β_2 subunit from light, and was stopped by the addition of soybean trypsin inhibitor (Sigma) in slight molar excess over the trypsin. Unless otherwise

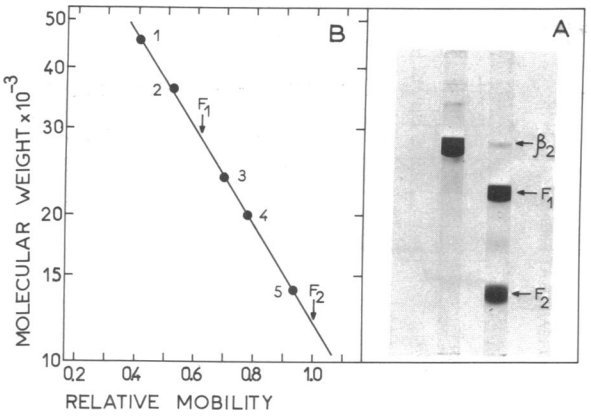

FIG. 1. Gel electrophoresis of trypsin-treated β_2 subunit. (A) The β_2 subunit (1 mg/ml) was treated with trypsin and the proteolysis stopped as described under *Materials and Methods*. Untreated (left) or trypsin-treated (right) protein (100 μg) was analyzed by electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate. (B) The molecular weight of the fragments was determined from their migration on 8% polyacrylamide gels in the presence of sodium dodecyl sulfate. The marker proteins were: (*1*) the β_2 subunit of tryptophan synthetase (45,000); (*2*) lactate dehydrogenase (35,000); (*3*) trypsin (24,000); (*4*) soybean trypsin inhibitor (20,000); and (*5*) lysozyme (14,000).

stated, the concentration of trypsin used was 1% (wt/wt of β_2) and the proteolysis lasted for 30 min.

Gel Electrophoresis. Disc electrophoresis on polyacrylamide gels was performed in the presence of sodium dodecyl sulfate according to Shapiro *et al.* (15). For quantitative colorimetric visualization of proteins, the method of Fenner *et al.* (16) was used.

Analytical Centrifugations. The centrifugations were done in a Centriscan 75 (M.S.E., England) analytical ultracentrifuge using the ultraviolet absorption scanning system at 280 nm. Molecular weights were measured by sedimentation equilibrium at high speed according to Yphantis (17). The partial specific volume used for calculations was 0.738 ml/g (18).

Radioactivity Measurements and Equilibrium Dialysis. [^{3}H]Pyridoxal-*P* was prepared according to the method of Raibaud and Goldberg (19). Polyacrylamide gels containing labeled protein were sliced and radioactivity was determined as described by Tishler and Epstein (20). Equilibrium dialysis was done on 0.4-ml samples (0.4 mg/ml of protein) equilibrated for 20 hr at room temperature and in the dark against 16 ml of 10 μM [^{3}H]pyridoxal-*P* in 0.1 M potassium phosphate, pH 7.8, 2 mM EDTA, and 5 mM 2-mercaptoethanol. The radioactivity of the samples was measured after deproteinization with 3% trichloroacetic acid, taking into account a quenching of 4% by the trichloroacetic acid.

The radioactivity was measured on 50-μl aliquots in 10 ml of Bray's mixture with an Intertechnique SL 30 scintillation counter.

Absorption and Fluorescence Measurements. Absorption spectra were recorded on a Cary 17 spectrophotometer. Fluorescence measurements were performed with a double monochromator CGA (DC 3000/1) spectrofluorimeter equipped with a Servotrace PE (Sefram, Paris) recorder. The absorbance of the sample throughout the excitation range was kept below 0.1 to minimize internal filter effects. In the spectra reported, the fluorescence of the solvent has been subtracted.

RESULTS

With the aim of finding conditions under which its polypeptide chain would be split into large fragments, we subjected the β_2 subunit of tryptophan synthetase to various proteolytic treatments and analyzed the effect of the digestion on the size of the polypeptide chain by electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate. When holo-β_2 was treated with trypsin two protein fragments, F_1 and F_2, were obtained (Fig. 1A).

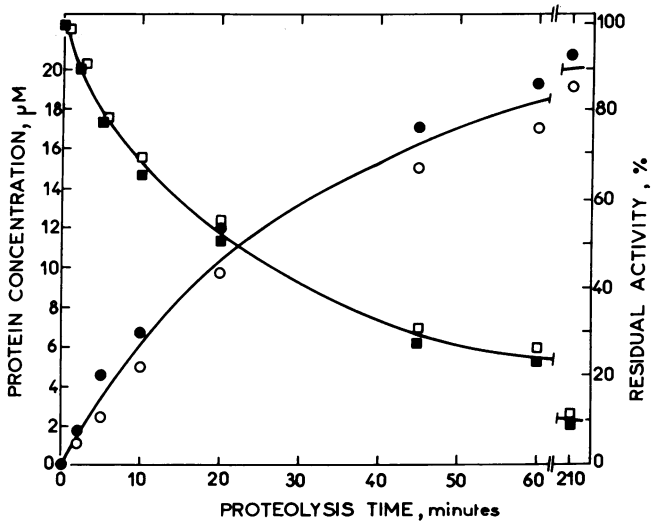

FIG. 2. Kinetics of proteolysis. A solution (1 mg/ml) of the β_2 subunit in buffer A was treated with 0.5 μg/ml of trypsin at 20°. At the times indicated on the abscissa, 40-μl aliquots were removed and added to 160 μl of the trypsin inhibitor at 5 μg/ml. Each aliquot was then assayed for activity and analyzed on sodium dodecyl sulfate gels. The amount of protein in each band was quantitatively determined and converted to molarity of polypeptide chain using the molecular weights obtained from Fig. 1. (● ○, and ■) Molarity of F_1, F_2, and β subunit, respectively. (□) L-Serine deaminase activity. The indole to tryptophan activity gave similar values.

Proteolysis of the β_2 subunit by trypsin

The degradation of the β_2 subunit by trypsin was therefore further investigated. First, the molecular weight of the two fragments was determined from their migration on the gels (Fig. 1B). The values were found to be 29,000 (fragment F_1) and 12,000 (fragment F_2), compared to 45,000 for the β chain (4).

Next, the kinetics of the degradation by trypsin was examined by measuring the disappearance of the intact monomers as a function of time; simultaneously, the residual activity of the treated β_2 subunit was determined. Fig. 2 shows that the loss of activity closely parallels the disappearance of the band corresponding to the intact monomer. In the same experiment, the kinetics of appearance of the two fragments were also followed. Fig. 2 shows that the disappearance of one intact protomer results in the stoichiometric formation of one of each fragment. Thus, it can be concluded that tryspin cleaves the β_2 subunit into two large, nonoverlapping, inactive fragments.

Quaternary structure of nicked β_2

The sedimentation velocity of the trypsin-treated enzyme was then studied by analytical centrifugation. The sedimentation pattern clearly showed the existence of two boundaries. The faster one corresponded to the majority of the absorbance, while a small amount of UV-absorbing material gave rise to a very slowly moving boundary. This material presumably corresponds to small peptides released during the proteolysis and could account for the difference between the 45,000 molecular weight of the intact protomers and the 41,000 of $F_1 + F_2$. The slowly sedimenting material was eliminated by filtering the

trypsin-treated β_2 subunit on a Sephadex G-75 column. Most of the protein was excluded from the gel and, when submitted to an analytical centrifugation, gave rise to a single, sharp and symmetrical boundary. This material (i.e., trypsin-treated β_2 freed from the slowly sedimenting peptides) thus appears as a homogeneous molecular species which will, hereafter, be called the "nicked β_2" protein.

The sedimentation coefficient of nicked β_2 was measured; the value obtained, $s_{20,w} = 4.6 \pm 0.1$ S, is close enough to that of the intact β_2 subunit (5.1 S) (12) to suggest that the fragments remain associated in the nicked protein. To be certain of this, we subjected the nicked β_2 protein to centrifugation at equilibrium at 18,500 rpm and 20° for 20 hr. The protein appeared homogeneous, and the value obtained for its molecular weight was 83,000 ± 5,000, in perfect agreement with the assumption that nicked β_2 would be a tightly associated dimeric structure, each protomer containing one F_1 (29,000) and one F_2 (12,000) fragment.

Enzymatic properties of nicked β_2

We have shown (Fig. 2) that nicked β_2 is inactive in the L-serine deamination reaction. It has also been observed that nicked β_2 is inactive in the indole to tryptophan reaction under all conditions tested [i.e., in the absence or presence of the α subunit or 2 M ammonium sulfate, known to activate β_2 (10)].

Ligand binding by nicked β_2

Even though inactive, the nicked protein might have kept the ability to bind some of the ligands of the β_2 protein. Therefore, the binding reactions of the α subunit, pyridoxal-*P*, L-serine, and indole to nicked β_2 have been successively investigated.

Association with the α Subunit. Several inactive immunologically crossreacting proteins, produced by mutants affected in the gene coding for β_2, are yet able to increase by a factor up to 100 the ability of the α subunit to catalyze the hydrolysis of indoleglycerol-phosphate (12). Therefore, the capacity of nicked β_2 to activate α was investigated as a possible probe for interactions between these two proteins. However, no activation of α by nicked β_2 could be detected under conditions where native β_2 exerted a 100-fold activation.

A more direct approach was used to test the interactions between α and nicked β_2. Three solutions (α, nicked β_2, and a mixture of both) were subjected to high-speed centrifugation (Fig. 3). The sedimentation pattern of the mixture showed two boundaries which, in sedimentation coefficient and amount of material, appeared to be identical to those of the free α and nicked β_2. As shown by Goldberg *et al.* (21), an interaction between the components in the mixture would have resulted in a decrease of the height of the slower boundary and an increase of the height and sedimentation coefficient of the faster boundary. In a control experiment, performed under the conditions of Fig. 3, such was indeed the case, when β_2 was used instead of nicked β_2. It can therefore be concluded that nicked β_2 has lost its ability to form a stable complex with the α subunit.

Binding of Pyridoxal-*P*. When pyridoxal-*P* was removed from the trypsin-treated protein and the proteolysis was continued, the fragments were degraded much more rapidly than in the presence of the cofactor. This protection by pyridoxal-*P* against further degradation of the nicked protein already suggested that the cofactor still binds to nicked β_2.

This was confirmed by a direct binding determination performed as follows. A sample of apo nicked-β_2 was prepared by incubation of nicked β_2 with 2 mM hydroxylammonium chloride followed by a dialysis with several changes against 0.1 M potassium phosphate, pH 7.8, 2 mM EDTA, and 5 mM 2-mercaptoethanol. A parallel experiment, performed with [^{3}H]pyridoxal-*P*-labeled nicked β_2, showed that less than 1% of the cofactor remained bound to the protein after this resolution treatment. The apo nicked-β_2 thus obtained was subjected to an equilibrium dialysis against the radioactive cofactor. The nicked protein could bind 0.7 molecule of pyridoxal-*P* per protomer while, under these conditions, the native β_2 subunit had 0.9 binding site per protomer. The nicked β_2 protein therefore appears to have retained nearly 80% of the coenzyme binding sites initially present on β_2.

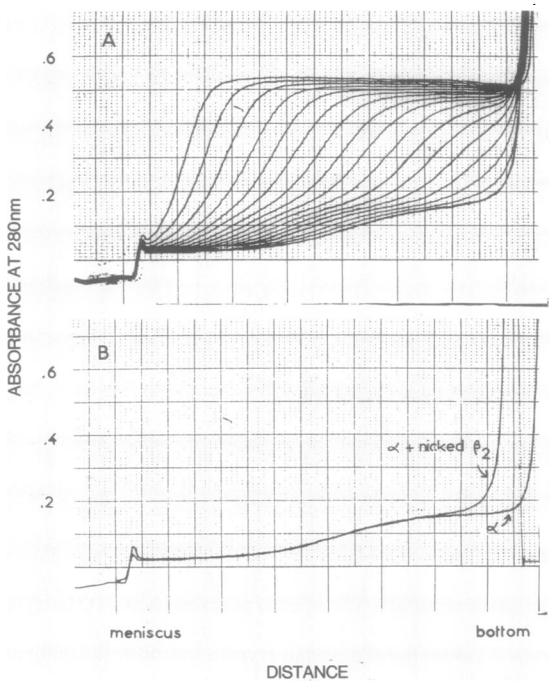

FIG. 3. Sedimentation of a mixture of nicked β_2 with the α subunit. α (0.40 mg/ml) and a mixture of nicked β_2 (0.81 mg/ml) and α (0.40 mg/ml) were centrifuged at 55,000 rpm in 0.1 M potassium phosphate buffer, pH 7.8, containing 2 mM EDTA, 5 mM 2-mercaptoethanol, and 50 μM pyridoxal-*P*. (A) Sedimentation of the mixture; 10-min interval between each scan. (B) Comparison of the slow boundary in the two cells; the sedimentation diagram was recorded, in each cell, 180 min after full speed was reached. At that time, when a mixture of α and β_2 was sedimented under identical conditions, all the proteins had reached the bottom and the absorbance was zero throughout the cell.

In order to identify the fragment that carries the residue involved in the Schiff base with the cofactor (6), we prepared nicked β_2 from a sample of β_2 labeled with [^{3}H]pyridoxal-*P*. The nicked protein was then treated with 10 μl/ml of sodium borohydride (5 mg/ml in 0.2% NaOH), and subjected to an electrophoresis in sodium dodecyl sulfate. All the label was found at the level of fragment F_1, thus demonstrating that this fragment carries the residue that forms the covalent bond with pyridoxal-*P*.

Since the binding of the cofactor to native β_2 results in a characteristic absorption spectrum (6), the spectral properties of the nicked holo-β_2 were also investigated. The absorption spectrum (Fig. 4A), with two maxima at 335 and 417 nm characteristic of protein-bound pyridoxal-*P*, is similar to that of native holo-β_2 (6). The nicked holo-β_2 also exhibits fluorescence properties similar to those of the native enzyme (22): an emission maximum close to 500 nm and an excitation maximum at 412 nm (Fig. 4B).

In conclusion, the nicked β_2 has retained the ability to bind

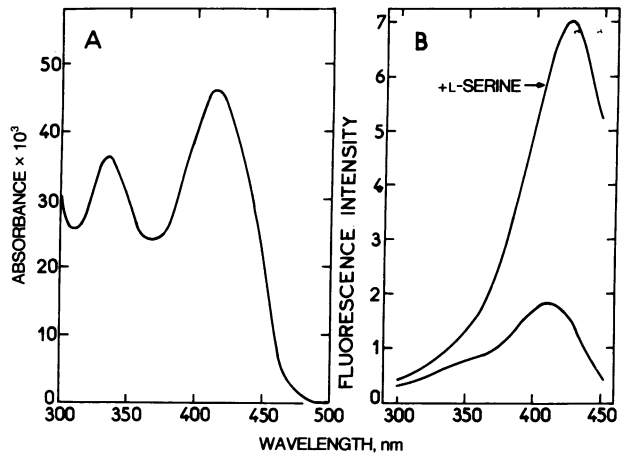

FIG. 4. Spectral properties of nicked β_2. (A) Absorption spectrum of nicked β_2 at 0.40 mg/ml. (B) Fluorescence excitation spectrum of nicked β_2 at 0.14 mg/ml in the absence or presence of 0.1 M L-serine. $\lambda_{emission}$ = 510 nm; fluorescence intensity in arbitrary units. The buffer used was 0.1 M potassium phosphate buffer, pH 7.8, supplemented with 2 mM EDTA, 2 mM 2-mercaptoethanol, and 10 μM pyridoxal-*P*.

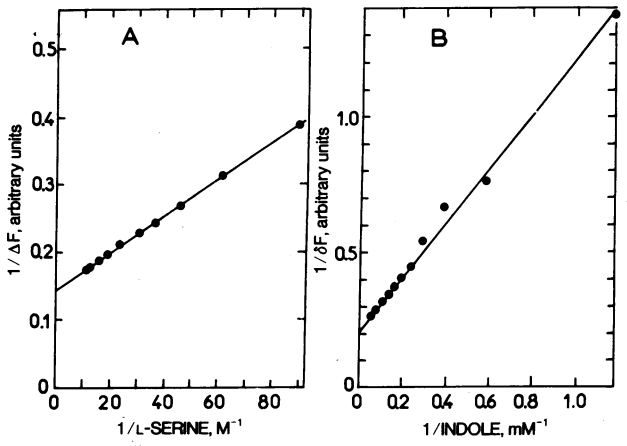

FIG. 5. Effect of L-serine and indole on the fluorescence of nicked β_2. Nicked β_2 (0.14 mg/ml) in 0.1 M potassium phosphate buffer, pH 7.8, 2 mM EDTA, 2 mM 2-mercaptoethanol, and 10 μM pyridoxal-*P* was supplemented with aliquots of 1 M L-serine (A) or with 0.1 M L-serine and aliquots of 1 M indole in ethanol (B). (A) Effect of L-serine. The increase in fluorescence intensity (ΔF) was measured as a function of L-serine concentration and is shown in double reciprocal coordinates. (B) Effect of indole. The decrease (δF) in the fluorescence of the "aqua"-band was measured as a function of indole concentration and is shown in double reciprocal coordinates. $\lambda_{excitation}$ = 440 nm; $\lambda_{emission}$ = 510 nm.

one pyridoxal-*P* per protomer via a Schiff base with a residue contained in fragment F_1 and in an environment which, according to the spectral properties of the bound cofactor, must be quite similar to that existing in the native enzyme.

Binding of L-Serine. The binding of L-serine to native holo-β_2 causes a striking increase in the fluorescence intensity and a red shift of the excitation maximum of the bound cofactor (22). This fluorescence band, called the "aqua-band" (22), is also exhibited by the nicked enzyme. Fig. 4B shows that the same is true for nicked β_2: when 0.1 M L-serine was added to the nicked holo-β_2, a 4-fold increase in the fluorescence intensity and a shift to 425 nm of the excitation maximum were observed.

Because the difference, ΔF, between the fluorescence of nicked β_2 in the absence and presence of L-serine is the largest when the sample is excited at 440 nm, this wavelength was chosen to measure the dissociation constant for L-serine. The fluorescence change, ΔF, was measured as a function of L-serine concentration (see Fig. 5A). Extrapolation of the straight line obtained in the double reciprocal plot yields the dissociation constant for L-serine: $K_d^{Serine} = 2 \times 10^{-2}$ M. Even though this value is significantly higher than that ($\simeq 10^{-3}$ M) determined, in a similar way, for native β_2 (22), it can be concluded that nicked β_2 has kept a binding site for L-serine.

Binding of Indole. The "aqua"-band exhibited by native β_2 in the presence of L-serine is quenched by the binding of indole (22). Similarly, Fig. 5B shows that the addition of indole to nicked β_2 in the presence of L-serine results in a decrease of the fluorescence of the "aqua"-band. Since results previously reported (22) demonstrated that indole and serine bind independently to native β_2, it can be assumed that the same is true for nicked β_2. Accordingly, the extrapolation of the straight line in Fig. 5B yields the dissociation constant for indole: $K_d^{Indole} = 6 \times 10^{-3}$ M. That this value is close to that, 3.7×10^{-3} M, reported for β_2 (22) clearly indicates that the binding sites for indole have been preserved in the nicked enzyme.

DISCUSSION

These experiments show that limited proteolysis of the β_2 subunit of tryptophan synthetase results in a nicked dimeric protein, the protomer of which contains two large fragments of the original polypeptide chain. Though inactive and unable to combine with the α subunit, the nicked enzyme has kept specific binding sites for the substrates and cofactor of the native protein (indole, L-serine, and pyridoxal-*P*). Moreover, the fluorescence properties characteristic of the enzyme-bound Schiff base between L-serine and pyridoxal-*P* (22) are also exhibited by the nicked enzyme. Therefore, the lack of activity of this protein must result from a block beyond this intermediate in the sequence of partial reactions described by Miles and Kumagai (23).

The inactivation of β_2 upon nicking may be explained in two ways: either some residue, essential for the catalysis and directly involved in the active site, has been released from the protein during trypsinolysis or the protein has undergone an overall change in conformation which affects its active site. If the latter assumption is correct, the change in conformation must be quite subtle since the binding sites for pyridoxal-*P*, indole, and L-serine are preserved. A complete characterization of these binding sites (e.g., inhibitors, specificity, dependence of the dissociation constants upon pH, temperature) and analysis of the formation and disappearance of the fluorescent intermediate on the inactive protein will help in the elucidation of the catalytic mechanism of the native enzyme by rapid kinetic methods (24).

The main interest of the nicked protein resides in the possibilities it offers for investigating the process of protein folding. While several fragmented proteins have been obtained (either by controlled proteolysis or by a genetic approach) and shown to retain some of their functional properties (25–32), very few have been used for folding studies either because the renaturation of even the unmodified enzyme could not be achieved (25, 29) or because the characterization of the isolated fragments would have been difficult because of the lack of a convenient structural probe. Thus, the confirmation of the "globule"-model proposed to account for the folding of *E. coli* β-D-galactosidase (33) required the very tedious and effort-consuming (but how elegant) immunological approach of Celada *et al.* (34). For nicked β_2, both difficulties are likely to be easily overcome, since the renaturation of intact β_2 has been described as

straightforward and easy (4) and since the protein carries many specific interaction sites that can serve as probes for the conformation of the "renatured" isolated fragments.

Indeed, preliminary experiments in our laboratory have already shown that the fragments F_1 and F_2 can be isolated in the presence of urea and renatured separately in a nondenaturing solvent, where they remain soluble. Moreover, it has also been observed that, upon mixing these "renatured" fragments, the fluorescence properties of the undenatured, nicked protein can be recovered (i.e., appearance of the "aqua"-band with L-serine and quenching by bound indole), thus showing that the "renatured" fragments are able to recombine and yield the native nicked β_2 subunit.

This result is to be compared with the genetic complementation experiments performed by Kida and Crawford (35) which strongly suggest the existence of two complementation groups situated on either side of locus 212 on the tryptophan synthetase B gene. It is possible that the F_1 and F_2 fragments might be closely related to these complementation groups, and it is therefore not unlikely that *in vitro* complementation might occur between a fragment and some crossreacting proteins obtained from chain termination or deletion mutants. If such were the case and if the isolated renatured fragments are shown to bind specific ligands, the "globule" model (33), which originated from complementation studies on β-D-galactosidase, could be extended to the β_2 subunit of tryptophan synthetase. This would strongly support the idea that the "domains" observed in proteins do correspond to intermediate structures in the folding of proteins. One then could consider that the results of Anfinsen's group on the structure of staphylococcal nuclease fragments (36) can account for the folding of a "globule." In turn, the specific noncovalent association of these "globules" within the polypeptide chain would be the last step in the achievement of the native conformation and would account for the frequent observation of domains in the structure of large proteins.

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