An Active Proteolytic Derivative of the α Subunit of Tryptophan Synthase. Identification of the Site of Cleavage and Characterization of the Fragments†

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ABSTRACT: Previous studies showed that the limited tryptic proteolysis of the $\alpha_2\beta_2$ complex of tryptophan synthase yields an active $\alpha_2\beta_2$ derivative which can be resolved into an active β_2 dimer and an active α derivative termed α' [Miles, E. W., & Higgins, W. (1978) *J. Biol. Chem. 253*, 6266–6269]. α' has now been resolved into two fragments, α-1 and α-2, by gel filtration in 6 M urea. A site of cleavage of the α chain at arginine-188 has been identified by amino acid analysis and N-terminal sequence analysis of the two fragments; α-1 is the amino-terminal part of the α chain and α-2 is the carboxy-terminal fragment. Circular dichroism spectra in the far-UV show that the separate α-1 and α-2 fragments have ordered structure after removal of urea. Further evidence that the α-1 fragment has an ordered, folded structure results from solvent perturbation and circular dichroism studies of tyrosyl residues. Whereas neither of the fragments alone has catalytic activity or the ability to bind the substrate analogue indolepropanol phosphate or the holo β_2 dimer, mixing of the two fragments restores those binding properties and catalytic activity. We conclude that the two fragments are separate folding domains which must interact to form functional enzyme. In addition, we conclude that arginine-188 must be readily accessible to trypsin in the $\alpha_2\beta_2$ complex, since cleavage at this bond is the predominant result of limited tryptic proteolysis of the $\alpha_2\beta_2$ complex.

Limited proteolysis can be a useful probe of protein structure and function. Studies of the protein fragments resulting from proteolysis may show which regions of the peptide chain are necessary for activity or proper folding (Anfinsen & Scheraga, 1975). For example, Högberg-Raibaud & Goldberg (1977a,b) have found that limited trypsin treatment of tryptophan synthase β_2 dimer yields an enzymatically inactive complex of two nonoverlapping fragments, F_1 and F_2, which can be separated under denaturing conditions and shown to refold independently. We have recently reported (Miles & Higgins, 1978; Higgins, 1978) that limited tryptic proteolysis of the $\alpha_2\beta_2$ complex of tryptophan synthase cleaves the α subunit and not the β_2 dimer. The product is an active, stable $\alpha'_2\beta_2$ complex which can be resolved into an active β_2 dimer and an active proteolytic derivative of α, termed α'. During gel electrophoresis under denaturing conditions, α' can be resolved into two fragments, α-1 and α-2.

In the present work we have separated the α-1 and α-2 fragments under denaturing conditions and have studied their chemical and physical properties. α-1 is shown to be the amino-terminal fragment; α-2 is the carboxy-terminal fragment. Although circular dichroism and solvent perturbation studies show that α-1 and α-2 are largely refolded after removal of denaturant, neither fragment alone can interact with a substrate analogue, indolepropanol phosphate, or with the β_2 dimer. In contrast, when the isolated, inactive fragments are mixed, the two fragments recombine to form the enzymatically active derivative α'.

Experimental Procedures

Materials. Pyridoxal phosphate and soy bean trypsin inhibitor were purchased from Sigma Chemical Co. Carboxypeptidases A, B, and Y were from Worthington Biochemical Corp. Indolepropanol phosphate was a generous gift of Dr. Kaspar Kirschner [see Kirschner et al. (1975b)]. 5,5′-Dithiobis(2-nitrobenzoic acid) was from Eastman. Urea (ultrapure) was a product of Schwarz/Mann. Dithiothreitol and *N,N*-bis(2-hydroxyethyl)glycine were from Calbiochem. [^{3}H]HCHO, 100 mCi/mmol, was from New England Nuclear Corp.

Enzymes. The $\alpha_2\beta_2$ complex of tryptophan synthase was prepared from 500 g wet weight of *Escherichia coli* strain *W3110 trpR⁻ cysB⁻ Δ trp LD102 trpB⁺ trpA⁺/F′ colVB cysB⁺ Δ trp LD102 trpB⁺ trpA⁺* [1] through the first DEAE-Sephadex eluate according to Adachi et al. (1974). It was further purified by a procedure of Kirschner.[2] The DEAE-Sephadex eluate (9 g of protein in 535 mL) was made 1.3 M in ammonium sulfate and applied to a 1-L column (5 × 50 cm) of Sepharose 4B equilibrated with 0.1 M potassium phosphate, pH 7.0, 2 mM EDTA, 0.02 mM pyridoxal phosphate, 1 mM dithiothreitol, and 1.3 M ammonium sulfate. The column was washed with 1 L of the equilibration buffer and eluted with a linear gradient between 1 L of equilibration buffer and 1 L of buffer containing no ammonium sulfate. A sharp peak of active $\alpha_2\beta_2$ complex (4 g of essentially pure enzyme in 500 mL) eluted about halfway through the gradient. The enzyme was concentrated by precipitation with ammonium sulfate, followed by dialysis against 0.05 M sodium *N,N*-bis(2-hydroxyethyl)glycine buffer, pH 7.8, containing 1.0 mM EDTA and 1.0 mM dithiothreitol (buffer A).

Proteolysis. The $\alpha_2\beta_2$ complex (about 500 mg in 14 mL of buffer A) was treated with 5 μg/mL TPCK–trypsin (Worthington) for 60 min at 22 °C; soy bean trypsin inhibitor (Sigma) was then added to 50 μg/mL.

Separation of Subunits. The $\alpha_2\beta_2$ complex was resolved by the method of Miles & Moriguchi (1977). Complex or trypsin-treated complex (about 500 mg in 14 mL of buffer A) was treated with 1 M KSCN and 0.01 M NH_2OH for 10 min

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[1] We are grateful to Dr. Charles Yanofsky for a gift of this strain.

[2] We are grateful to Dr. Kasper Kirschner for providing us with this procedure prior to publication.

at 22 °C, applied to a 5 × 40 cm column of Sephadex G-100SF[3] equilibrated with buffer A, and eluted at 30 mL/h, collecting 10-mL fractions. Apo-β_2 dimer was eluted in fractions 31–36; α subunit or α' (the product of proteolysis) was eluted in fractions 42–50.

Activity Assays. The synthesis of L-tryptophan from indole and L-serine was assayed by a modification of the spectrophotometric method of Faeder & Hammes (1970) at 37 °C in a Cary 118 spectrophotometer. The reaction mixture contained 0.1 M Tris-HCl buffer, pH 7.8, 0.18 M NaCl,[4] 0.2 mM indole, 0.04 M L-serine, and 0.05 mM pyridoxal phosphate. α subunit was assayed with excess β_2 dimer; β_2 dimer was assayed with excess α subunit. One unit of activity is the conversion of 0.1 μmol of substrate to product in 20 min at 37 °C; this is equal to a change in absorbance at 290 nm of 0.185 under the assay conditions described above.

Protein Assays. The specific absorbance used for the holo β_2 dimer was $E_{278nm}^{1\%} = 6.5$ (Hathaway & Crawford, 1970). The specific absorbance values of α subunit and its derivatives were determined as described under Results.

Spectra. Circular dichroism spectra were measured with a Cary Model 60 spectrophotometer with a Model 6001 circular dichroism attachment at 27 °C, using a computer system (Shapiro & Schultz, 1971). Scaling of the data and addition and subtraction of spectra and base lines were carried out by the computer. Mean residue ellipticity values, $[\Theta]_{mrw}$, were calculated by using the expression

$$[\Theta]_{mrw} = \frac{[\Theta]_{mrw}}{lc'10}$$

where Θ is the observed ellipticity, l is the path length in centimeters, and c' is the concentration in grams per milliliter; the units are degrees centimeter^2 decimole^{-1}. The mean residue molecular weight (mrw) for the α subunit was calculated to be 107.2 from the amino acid sequence of Li & Yanofsky (1973); the mean residue molecular weights of the α-1 and α-2 fragments were calculated to be 108.9 and 103.4 by using the amino acid composition based on the site of cleavage of the α chain (see Results). Circular dichroism spectra were made on protein solutions of 1–2 mg/mL in 0.1-cm path length cells in the near-ultraviolet (250–320 nm) and in 0.1-mm "sandwich cells" (Hellma) in the far-ultraviolet (200–260 nm). The maximum absorbance was below 1.4 in all cases.

Absorbance spectra, difference spectra, and solvent perturbation difference spectra were recorded in a Cary 118 spectrophotometer. Difference spectra were obtained by measuring the spectra in split cuvettes (Hellma).

Sulfhydryl Modification. Protein solutions (about 1 mg/mL in 1.0 mL of 0.1 M potassium phosphate, pH 7.5, containing 1 mM EDTA) were treated with 0.2 mM 5,5′-dithiobis(2-nitrobenzoic acid) at 37 °C for 30 min; the absorbance at 412 nm was monitored continuously. After 30 min each sample was treated with solid urea and additional buffer to give 2.0 mL of solution containing 8 M urea; the absorbance at 412 nm was recorded; the number of sulfhydryls was calculated by using an extinction coefficient for the thionitrobenzoic acid released of 13 600 M^{-1} cm^{-1} at 412 nm (Ellman, 1959).

Gel Electrophoresis. Polyacrylamide gel electrophoresis was performed in the presence of sodium dodecyl sulfate according to Laemmli (1970) or in the presence of urea (8 M) and sodium dodecyl sulfate (0.1%). The original procedure of Swank & Munkres (1971) was modified in order to make it more suitable for slab gel electrophoresis. Gels contained 12.5% acrylamide, 0.34% bis(acrylamide), and 8 M urea. The original reservoir solution (Swank & Munkres, 1971), containing 0.1% sodium dodecyl sulfate and 0.1 M H_3PO_4 adjusted to pH 6.8 with Tris, was used, and gels were run at 25–30 mA for 16–20 h at 22 °C.

Labeling α' with [^{3}H]HCHO. α' was labeled with [^{3}H]-HCHO by using the method of Rice & Means (1971). α' (5 mg in 2.5 mL of 0.2 M sodium borate, pH 9.0) was treated with 0.25 mL of 0.04 M [^{3}H]HCHO (New England Nuclear Corp.; 100 mCi/mmol) at 0 °C, followed in 30 s by four 50-μL sequential additions of sodium borohydride (5 mg/mL in H_2O); an additional 0.25 mL of the sodium borohydride solution was added after 1 min. The solution was dialyzed for 72 h against 0.05 M sodium borate buffer, pH 9.0. The final specific activity of [^{3}H]α' was 42 000 cpm/nmol.

Isolation of Tryptic Fragments. α' (28 mg) in 1.7 mL of *N*,*N*-bis(2-hydroxyethyl)glycine, pH 7.8, containing 6 M urea, 2 mM EDTA, and 2 mM dithiothreitol was combined with 0.2 mL of [^{3}H]α' (1.5 mg/mL; 1.4 × 10^6 cpm/mg). This solution was applied to a column (1.6 × 60 cm) of Sephadex G-100 (fine) previously equilibrated with 50 mM sodium *N*,*N*-bis(2-hydroxyethyl)glycine buffer (pH 7.8) containing 6 M urea, 2 mM EDTA, and 2 mM dithiothreitol. Fractions (2 mL) were assayed for protein by measuring absorbance at 278 mm, and 25-μL aliquots were counted for radioactivity. α-1 (fractions 18–27) was dialyzed against 50 mM sodium *N*,*N*-bis(2-hydroxyethyl)glycine buffer (pH 7.8) containing 2 mM EDTA and 2 mM dithiothreitol. α-2 (fractions 31–42) was desalted on Sephadex G-25 (1.6 × 90 cm) by using 0.1 N acetic acid. α-1 and α-2 were checked for purity for sodium dodecyl sulfate–polyacrylamide electrophoresis in the presence of urea (see Figure 1).

Amino Acid Analysis. Proteins and peptides (1–10 nmol) were hydrolyzed in vacuo in 6 N HCl (200–500 μL) at 105 °C for 24 h routinely and in certain cases for 24, 48, and 72 h. Some samples were oxidized with performic acid (Hirs, 1967) before hydrolysis. Hydrolysates were analyzed on a Beckman 121M amino acid analyzer using the single-column methodology[5] (Table I; see paragraph at end of paper regarding supplementary material).

Automatic Edman Degradation. Edman degradations were performed automatically by using a modified Beckman sequencer, Model 890B. The sequencer was equipped with a cold trap for the high vacuum and a large cup [see Fairwell & Brewer (1979)]. The coupling buffer was 0.1 M quadrol,[6] and double coupling was performed at the first residue. The PhNCS amino acid derivatives were identified by mass spectroscopy (Fairwell & Brewer, 1973), gas chromatography (Pisano et al., 1972), and high-pressure liquid chromatography (Zimmerman et al., 1977). High-pressure liquid chromatography was found to be most reliable for quantitation of PhNCS amino acids. α-1 peptide was prepared for sequence analysis by overnight dialysis against deionized water (Table II, supplementary material). α-2 peptide was desalted on Sephadex G-25 in 0.1 N acetic acid (Table III; supplementary

[3] The use of the superfine material appears to be essential for good separation.

[4] The omission of NaCl in our previous studies with this assay (Miles & Moriguchi, 1977; Miles & Higgins, 1978) led to lower specific activities than reported in this work.

[5] We are grateful to Barbara F. Torain, Laboratory of Chemistry, National Institute of Arthritis, Metabolism, and Digestive Diseases, for the amino acid analyses.

[6] Abbreviations used: quadrol, *N*,*N*,*N*′,*N*′-tetrakis(2-hydroxypropyl)ethylenediamine; PhNCS, phenylthiohydantoin; LC, high-pressure liquid chromatography.

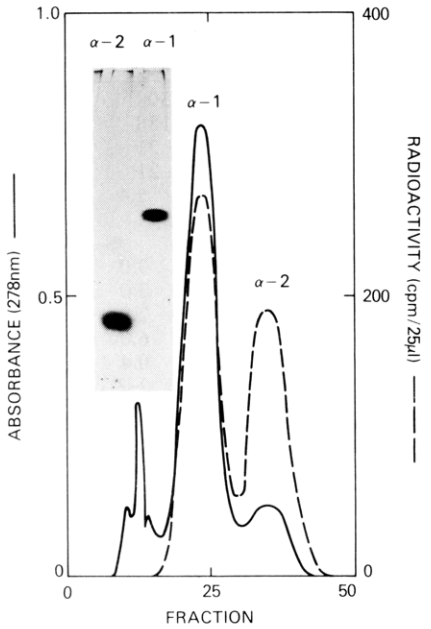

FIGURE 1: Separation of [^{3}H]α' into α-1 and α-2 by gel filtration in the presence of 6 M urea. Conditions of labeling and gel filtration are described under Experimental Procedures. The inset shows sodium dodecyl sulfate–urea–polyacrylamide electrophoresis of pooled α-1, fractions 18–27 (10 μg, 0.5 nmol), and α-2, fractions 31–42 (35 μg, 4.4 nmol), after staining with Coomassie Blue. The observation that the α-2 peak shows a much higher ratio of radioactivity to absorbance at 278 nm than does the α-1 peak probably reflects the much lower content of tyrosine in α-2 (one residue in α-2 vs. six residues in α-1) and the nearly equal number of lysyl residues which can be labeled with [^{3}H]HCHO (see Table I in supplementary material).

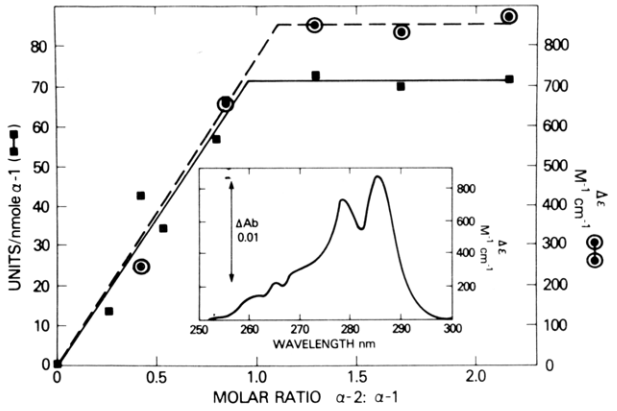

FIGURE 2: Effect of interaction of the α-1 and α-2 fragments on the enzymatic activity and on the difference absorbance. α-1 fragment (0.0225 mM in 0.1 M potassium phosphate, pH 7.8, containing 1 mM EDTA and 1 mM dithiothreitol) was treated with a series of aliquots of α-2 fragment (0.64 mM in water) to give the molar ratios indicated. Tryptophan synthase activity was assayed with excess β_2 subunit (see Experimental Procedures) and is plotted as units per nanomole α-1 fragment against the molar ratio of α-2/α-1 (■). Difference spectra of the mixed solutions of α-1 and α-2 (2.0 mL) were recorded in a split cuvette with a 0.88-cm path length against a matched cell in which equivalent amounts of α-1 and α-2 fragments were on the separate sides of the split cuvette. The molar difference absorbance at 286 nm was calculated from the molar concentration of the α-1 fragment (0.021 mM) and is plotted against the molar ratio of α-2/α-1 (⊙). The inset shows the actual difference spectrum of a solution containing 0.021 mM α-1 and 0.046 mM α-2.

material). Both peptides were lyophilized and dissolved in *N*,*N*-dimethylallylamine for transfer into the reaction cup.

Carboxypeptidase Treatment. Treatment with carboxypeptidase Y using the method of Hermodson et al. (1972) and with carboxypeptidase B and A by the method of Goldsmith & Konigsberg (1977) was followed by amino acid analysis.

Results

Isolation of Proteolytic Derivatives of the α Subunit. An active, proteolytic derivative of the α subunit, termed α', is isolated from trypsin-treated $\alpha_2\beta_2$ complex by gel filtration on Sephadex G-100 (superfine) as described under Experimental Procedures. Gel filtration of ^{3}H-labeled α' in 6 M urea (see Experimental Procedures) yields two main peaks which exhibit both absorbance and radioactivity (Figure 1). Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and 8 M urea (inset to Figure 1) shows that each peak contains a pure fragment. Urea is removed from the pooled fractions of each peak as described under Experimental Procedures.

Amino Acid Composition and Sequence of Each Fragment. The observed amino acid compositions of the α-1 and α-2 fragments are close to the calculated compositions of the two fragments containing residues 1–188 and 189–268, respectively, which would be obtained by a single cleavage at residue 188 in the published primary structure of the α subunit (Li & Yanofsky, 1972).[7] The values for lysine (7) and arginine (1) in the α-2 fragment are unusual and highly characteristic of this region of the α chain. The N-terminal amino acid sequence data for the first 15 residues of α-1 (200 nmol) and for the first 17 residues of α-2 (300 nmol) have been determined.[7] α-1 has the same N-terminal sequences as the published sequence for residues 1–15 of the α subunit (Li & Yanofsky, 1972); the N-terminal sequence of α-2 is identical with the published sequence of residues 188–205 and is consistent with cleavage between residues 188 and 189.

Treatment of α-1 with either carboxypeptidase B or carboxypeptidase Y released 1 mol of arginine (see Experimental Procedures). Treatment with a mixture of carboxypeptidase A plus carboxypeptidase B, or with carboxypeptidase A following release of 1 mol of arginine by carboxypeptidase B, gave no release of asparagine or glutamate (residues 187 and 186, respectively). Increased amounts of carboxypeptidase A or Y led to nonspecific cleavage of α-1.

Table IV shows some physical and chemical properties of the α subunit and its derivatives.

Reconstitution of Active α' from the Separated Fragments. Although we have previously shown that α' is active in reactions catalyzed by the α subunit and in stimulating reactions catalyzed by the β_2 subunit, the separate α-1 and α-2 fragments showed no significant stimulation of the β_2 subunit in the synthesis of L-tryptophan from indole and L-serine (see Experimental Procedures).

Titration of the α-1 fragment by the α-2 fragment (Figure 2) resulted in maximum activity at a 1:1 molar ratio of the two fragments; each fragment in this experiment had a specific activity of about 70 units/nmol in the presence of the complementary fragment and the β_2 subunit. By comparison, the α subunit had a specific activity of 145 units/nmol, and the α' derivative had an activity of 92 units/nmol.

Titration of the α-1 fragment by the α-2 fragment also results in a difference absorbance at 286 nm which is maximum when a molar equivalent of α-2 fragment is added to α-1 fragment. The shape of the difference absorbance spectrum (inset, Figure 2) is similar to that of the solvent perturbation curves of the α subunit and its derivatives [data not shown, but see Table IV and Herskovits (1967)] and suggests that one or more tyrosyl residues exposed in the

[7] The tables showing these data have been deleted at the suggestion of a reviewer in order to save space but are available as supplementary material.

Table IV: Physical and Chemical Properties of the α Subunit and Its Derivatives

subunit or derivative	α	α′	α-1	α-2
M_r[a]	28 800	28 800	20 500	8300
no. of residues[a]	268	268	188	80
% α helix[b]	44	44	31	15
% α helix[c]	26	29	21	22
$E_{278nm}^{1\%}$ or $E_{276nm}^{1\%}$[d,e]	4.5	4.5	5.4	1.8
				1.4
				av 1.6[f]
total SH calcd[a]	3.0	3.0	3.0	0
available SH found[g]	0.94	1.3	3.0	nd[j]
total SH found[h]	3.0	2.9	2.9	nd[j]
total tyrosine calcd[a]	7.0	7.0	6.0	1.0
fraction of tyrosine exposed[i]	0.35 ± 0.1	0.4 ± 0.1	0.4	1.4
approximate no. of residues of tyrosine exposed	2–3	2–3	2–3	1

[a] Calculated from the known amino acid sequence (Li & Yanofsky, 1973) and from the single point of cleavage at 188 established in this work. [b] Data from Figure 5A. The percent helix was calculated by the method of Chen et al. (1972):

$$\text{percent helix} = \left(\frac{[\Theta]_{mrw}^{222nm} + 2340}{-30300}\right)100$$

[c] Data from Figure 5A. The percent helix was calculated by the method of Greenfield & Fasman (1969):

$$\text{percent helix} = \left(\frac{[\Theta]_{mrw}^{208nm} + 4000}{-29000}\right)100$$

[d] The specific absorbance at 278 nm (α, α′, α-1) or at 276 nm (α-2) was determined by measuring the absorbance spectrum of a solution of the protein in 0.1 M potassium phosphate, pH 7.8, containing 1 mM EDTA before and after lyophilization and redissolving in an equal volume of 8 M urea. The concentration of the protein in both solutions was calculated from the absorbance of the protein in urea by using the known molecular weight and number of tyrosines per mole and the molar extinction coefficient of tyrosine residues at 275.5 nm in the unfolded protein in 8 M urea at pH 7.5 (Edelhoch, 1967); the α subunit contains no other residues (tryptophan or cystine) which absorb at 277.5 nm. Although Edelhoch (1967) used 6 M guanidine hydrochloride, we have used 8 M urea, since Yutani et al. (1977) have concluded from far-UV circular dichroism spectra that the α subunit is completely denatured in 8 M urea. [e] The specific absorption of α-2 was also determined by amino acid analysis of acid hydrolysates of a solution of known absorbance; norleucine was included as an internal standard. [f] An average of the specific absorption determined by the two methods (*d* and *e*), $E_{278nm}^{1\%} = 1.6$, was used for calculations in this work. [g] Taken as the number of SH reacted after incubation with 5,5′-dithiobis(2-nitrobenzoic acid) for 10 min at 37 °C. See Experimental Procedures and Figure 5. [h] Determined with 5,5′-dithiobis(2-nitrobenzoic acid) in 8 M urea. See Experimental Procedures and Figure 4. [i] The perturbation of tyrosyl residues by 20% glycerol in proteins dissolved in buffer (0.1 M potassium phosphate, pH 7.5, containing 1 mM EDTA) or in buffer containing 8 M urea was determined according to Herskovits (1967). The fraction of tyrosyl residues exposed was calculated as described by using a factor calculated from a model experiment using *N*-acetyl-L-tyrosine ethyl ester (Schwartz/Mann). Values for α and α′ are the average of three experiments; values for α-1 and α-2 are from a single experiment. [j] nd, not determined.

separate fragments become buried in a more hydrophobic environment as a result of subunit association.

Environment of Tyrosyl Residues in α Subunit and Derivatives. Circular dichroism spectra in the near-UV of the α subunit, α′, and a mixture of α-1 and α-2 fragments (parts A, B, and D of Figure 3, solid curves) are closely similar to each other and to those reported by Heyn & Weischet (1975) for the α subunit alone. These curves show strong negative ellipticity bands at 261 and 269 nm, which are characteristic of phenylalanine residues in proteins with ordered structure, and positive ellipticity bands at 276 nm and 283 nm, which are characteristic of tyrosyl residues in proteins with ordered structure (Kay et al., 1974). The circular dichroism spectrum of α-1 (Figure 3C, solid curve) is very different from that of α in the region 276–285 nm where a large negative band is observed. This suggests that tyrosyl residues may be in a very different environment in the proteolytic fragment α-1. Although the ellipticity spectrum of α-1 is qualitatively similar to that of the α subunit in 6 M urea (data not shown), the bands at 261, 268, and 280 nm in α-1 are 2–3 times greater in magnitude in α-1 than in the denatured α subunit. This is further evidence that aromatic residues in α-1 exist in an asymmetric environment in an ordered protein structure. α-2 alone has only two weak negative ellipticity bands at 261 and 269 nm (Figure 3C).

The number of tyrosyl residues exposed to solvent in the α subunit and its dervatives has been determined by measuring the perturbation of the spectra of these residues by 20% glycerol by using the method of Herskovits (1967). The results (Table IV) indicate that only two to three tyrosyl residues are exposed in α, α′, and α-1, whereas the single tyrosyl residue in α-2 is fully exposed.

Binding of Indolepropanol Phosphate to the α Subunit and Derivatives. The interaction of the α subunit and derivatives with the substrate analogue indolepropanol phosphate has been determined by circular dichroism studies in the near-UV (Figure 3) and by UV difference spectra (Figure 4). Addition of indolepropanol phosphate to the α subunit (Figure 3A) results in a large increase in negative ellipticity which has been shown by Heyn & Weischet (1975) to be due to binding of the analogue. The concentration of indolepropanol phosphate used in these experiments (100 μM) was 2 times the dissociation constant for indolepropanol phosphate (48 μM) reported by Heyn & Weischet (1975). Whereas α′ (Figure 3B) and a mixture of α-1 and α-2 (Figure 3D) show similar changes upon addition of indolepropanol phosphate, the α-1 fragment alone shows only a very small change upon addition of indolepropanol phosphate. This result, which suggests that α-1 alone binds indolepropanol phosphate either weakly or not at all, is confirmed by UV difference spectra (Figure 4). Interaction of indolepropanol phosphate with the α subunit produces a striking UV difference spectrum as previously shown by Kirschner & Wiskocil (1972). Similar difference spectra are obtained with α′ and a mixture of α-1 and α-2 fragments but not with the separate α-1 and α-2 fragments.

Far-UV Circular Dichroism Studies. Mean residue molecular weight and molar ellipticity curves of the α subunit and its derivatives (parts A and B of Figure 5, respectively)

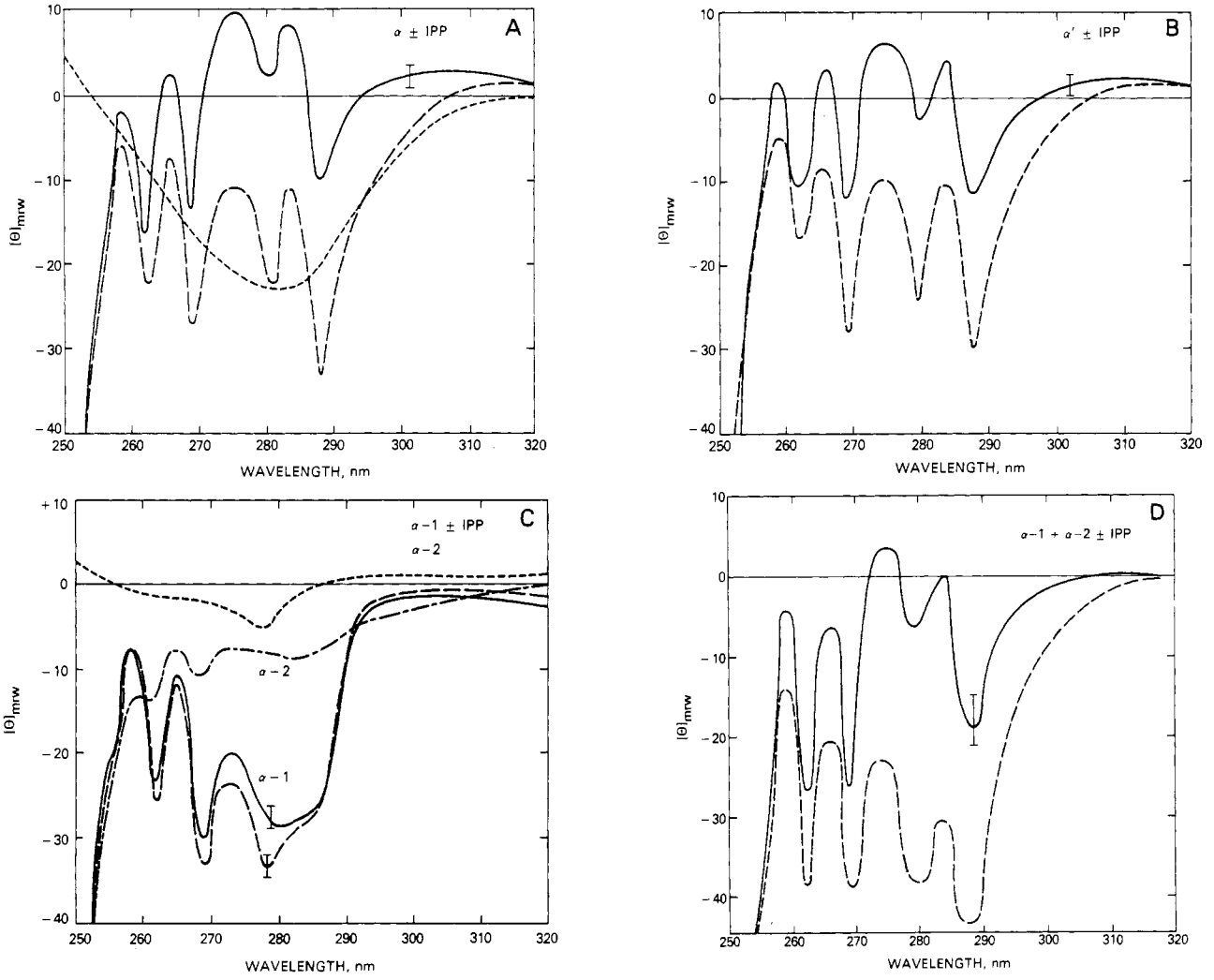

FIGURE 3: Near-UV circular dichroism spectra of the α subunit and its derivatives. Spectra of α subunit and derivatives (1–2 mg/mL in 0.05 M Tris-HCl, pH 7.8, containing 1.0 mM EDTA and 0.2 mM dithiothreitol) were recorded before (—) and after (– – –) addition of indolepropanol phosphate to a final concentration of 0.1 mM. (A) α subunit. (B) α'. (C) α-1 and α-2 (– · – · –) (indolepropanol phosphate was not added to α-2). (D) α-1 mixed with α-2 in a cell with a 1.0- or 0.88-cm path length. The difference between spectra plus indolepropanol phosphate (IPP) and spectra minus IPP is shown by (---) in (A) and (C). $[\Theta]_{mrw}$ is the mean residue ellipticity in degrees centimeters^2 decimole^{-1}.

show that all species have ordered structures. The percent α-helix estimated from these data by two methods [Chen et al. (1972) and Greenfield & Fasman (1969)] is shown in Table IV. The finding that the spectrum of the mixed α-1 and α-2 fragments differs slightly from the added sum of the spectra of the two separate fragments (dotted line in Figure 4B) may show that a small conformational change occurs upon mixing. The observation that the percent α-helix in the mixed α-1 and α-2 fragments is lower than that in the original α or α' may be due to the presence of some inactive material in the fragments (see Discussion).

Sulfhydryl Modification of the α Subunit and Its Derivatives. Whereas 5,5′-dithiobis(2-nitrobenzoic acid) reacts relatively slowly with one or two sulfhydryl residues of α, α', and α-1 mixed with α-2, it reacts rapidly with all three sulfhydryl residues in α-1 alone (Figure 6 and Table IV). Addition of 8 M urea results in complete modification of three sulfhydryl residues in each derivative.

Association of α and Its Derivatives with the Holo β_2 Subunit. Interaction of the α and holo β_2 subunits can be conveniently detected by difference spectra between the mixed and unmixed subunits (Kirschner et al., 1975a), which show peaks at 410, 278, and 285 nm (Figure 7, top curve). Similar curves are obtained for analogous experiments with holo β_2 and α' or α-1 previously mixed with α-2. In contrast, mixing the separate α-1 or α-2 fragment with holo β_2 gives no significant spectral change.

Discussion

Identification of the Site of Cleavage. The present work shows that trypsin cleaves the α subunit in the $\alpha_2\beta_2$ complex at arginine-188. This conclusion is based on the findings that α-2 has a homogeneous amino-terminal sequence with the correct primary sequence analysis for residues 189–206 and the expected amino acid analysis for this sequence. The presence of a single arginine in the α-2 fragment establishes that α-2 contains at least residues 189–267 since residue 267 is the only arginine in this sequence. Primary sequence analysis of α-1 demonstrates that it has the same amino-terminal sequence as the α subunit. The conclusion that α-1 contains residues 1–188 is supported by the amino acid analysis, by the identification of carboxy-terminal arginine, and by the finding that α-2 has a homogeneous amino-terminal sequence starting at residue 189. The failure of carboxypeptidase digestion of α-1 to yield the penultimate residues expected for cleavage at arginine-188 (asparagine-187 and glutamate-186) is probably due to the known slow release of these residues by carboxypeptidase A (Ambler, 1972a); the influence of the penultimate residue on the rate of release of the carboxy-terminal residue has been documented (Ambler, 1972b).

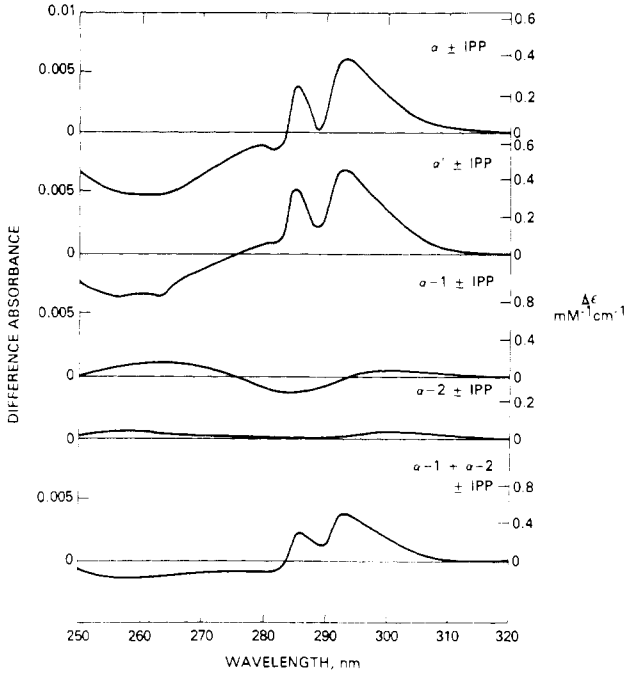

FIGURE 4: Difference spectra of indolepropanol phosphate (IPP) bound to the α subunit of tryptophan synthase and its derivatives. Difference spectra were measured in split cuvettes with a total path length of 0.88 cm by using 50 mM sodium *N*,*N*-bis(2-hydroxyethyl)glycine buffer, pH 7.8, containing 1.0 mM EDTA, 0.2 mM dithiothreitol, and 0.18 M NaCl. The final concentrations after mixing were 0.112 mM indolepropanol phosphate, 2500 units/mL, α, α', or α-2, and 1250 units/mL α-1 or α-1 previously mixed with a twofold excess of α-2. The molar concentration of each solution and the difference in molar extinction were calculated from the number of units of enzyme activity, assuming that fully active α subunit or derivative contained 145 units/nmol.

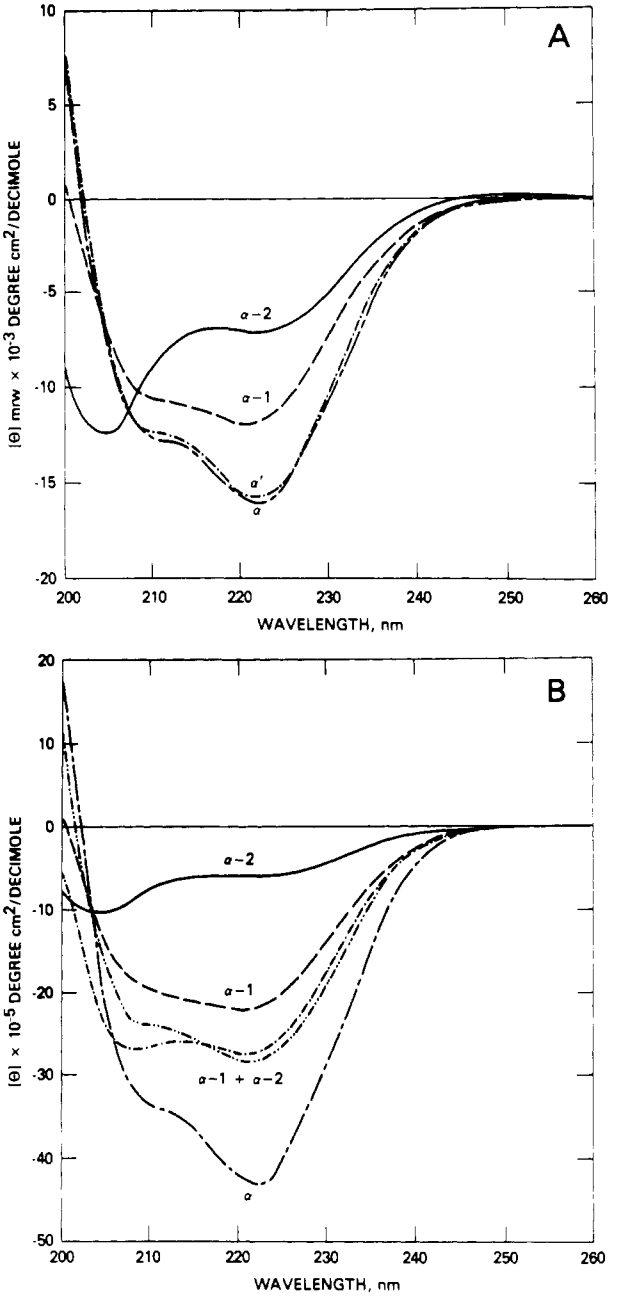

FIGURE 5: Far-UV circular dichroism spectra of the α subunit and its derivatives. Spectra of solutions as shown in Figure 3 were recorded in cells with a 0.1-mm path length. (A) Mean residue ellipticity curves in degrees centimeters^2 decimole^{-1}. (B) Molar ellipticity curves in degrees centimeters^2 decimole^{-1}. The dashed curve (- - - -) in (B) is the added sum of molar ellipticity curves for α-1 and α-2. The curve (- · - · -) shows a spectrum of mixed α-1 and α-2.

However, in the absence of a definite carboxy-terminal sequence for α-1, we cannot rule out the possibility that tryptic digestion has also occurred at additional sites on the amino-terminal side of arginine-188.

An earlier suggestion (Higgins, 1978) that the site of cleavage was in the amino-terminal region of the α chain is shown to be incorrect by the current work. The error resulted from the use of the gel electrophoresis method of Swank & Munkres (1971) to determine the molecular weights of CNBr peptides. This method, although excellent for separating and detecting low molecular weight peptides, is not reliable for determining the molecular weights of some peptides in this size range (Swank & Munkres, 1971). Conclusions based on this method also led to a low estimate of 3600 for the molecular weight of the α-2 fragment (Miles & Higgins, 1978), which is shown in the current work to be 8300. The calculated molecular weight of the α-1 fragment (20 500) agrees well with molecular weights of 20 000 and 23 000 estimated in our previous report (Miles & Higgins, 1978).

Evidence for Independent Folding Domains. The identification of the site of limited tryptic proteolysis of the α subunit increases our knowledge of the secondary structure of the α subunit and its mechanism of folding. A site susceptible to proteolysis would be expected to be at a bend or loop and not in a region with highly ordered structure. In the case of the β_2 subunit of tryptophan synthase, limited proteolysis has been shown to occur in a region between two independently folding regions or domains (Högberg-Raibaud & Goldberg, 1977a,b). An examination of the refolding of the separately isolated fragments of the α subunit shows that each has ordered structure as judged by circular dichroism in the far-UV (Figure 5). The observation that there is only a small difference between the circular dichroism spectrum of the mixed fragments (α-1 plus α-2) and the added sum of the spectra of the two fragments is evidence that only a small change in secondary structure or conformation occurs after mixing the two fragments. Thus, the two fragments appear to fold independently and may be considered to be independent domains by this criterion.

The finding that the circular dichroism spectrum (Figure 5) of the mixed fragments (α-1 plus α-2) is considerably different from the spectrum of α and α' even though the activities of α' and of α-1 plus α-2 are similar requires interpretation. One possible explanation is that α' is a mixture of some active and some inactive material with both forms

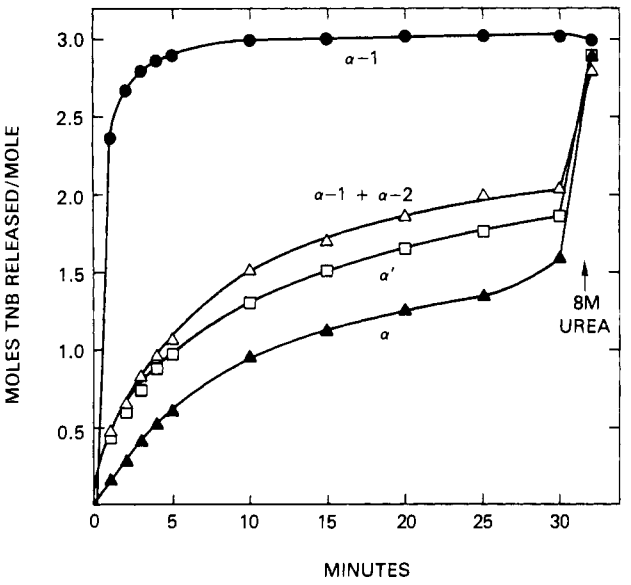

FIGURE 6: The reaction of α subunit and its derivatives with 5,5'-dithiobis(2-nitrobenzoic acid). Derivatives were treated as described under Experimental Procedures. The release of TNB (thionitrobenzoic acid) is plotted as a function of time. The curve labeled α-1 + α-2 was a mixture of α-1 and α-2.

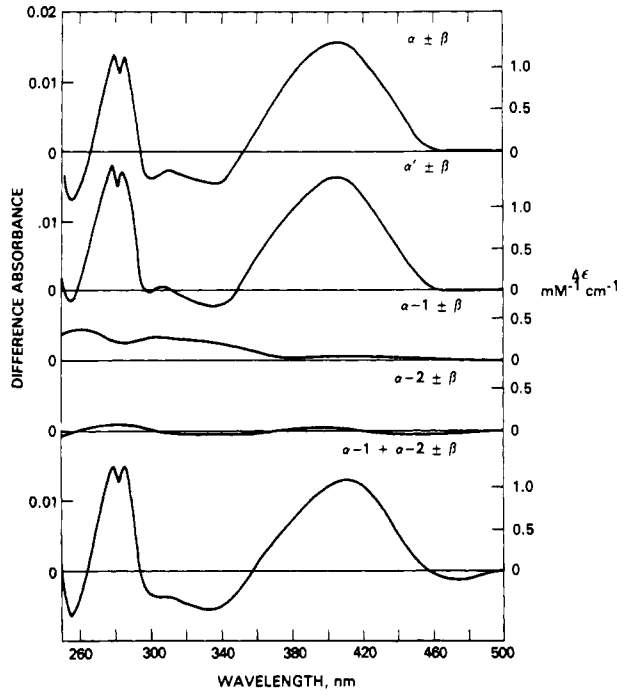

FIGURE 7: Difference spectra of holo β_2 subunit bound to the α subunit of tryptophan synthase and its derivatives. Difference spectra were measured by using the cuvettes and buffers described in Figure 4 except that holo β_2 was dialyzed against buffer also containing 0.02 mM pyridoxal phosphate. The final concentrations after mixing were 0.61 mg/mL holo β_2 (1270 units/mL) and a slight excess (1500 units/mL) of α or derivative. The difference in the molar extinction coefficient was calculated from the molar concentration of the holo β_2 subunit.

having unaltered folded structure prior to unfolding by urea. This situation might result if trypsin had nicked the chain in some molecules at a single location with no loss of activity but had nicked other molecules at two or more locations with complete loss of activity. Consistent with this interpretation is our finding of some variation in the specific activity of α' prepared under various conditions. Whereas α' prepared by limited proteolysis for 20 min had a specific activity equal to 85% of that of α (Miles & Higgins, 1978), α' prepared by using 60 min of proteolysis in the larger scale preparation reported in this work has a specific activity equal to about 63% of that of α. If the fragments produced by a single site of cleavage were able to refold after urea treatment to give active enzyme with the same amount of α helix as the native α, but the enzyme cleaved at more than one site was not able to refold after urea treatment, spectra similar to those in Figure 2 would be expected. Our recent finding that α-1 contains some higher molecular weight forms or aggregates which do not associate with α-2 indicates that α-1 is heterogeneous and contains some molecules which do not properly refold. Removal of these aggregates may yield homogeneous α-1 which will exhibit a higher percent α helix and a higher specific activity when recombined with α-2. We plan to do this as part of future, more detailed experiments on the refolding of α-1 and α-2.

Comparative studies of the environment and accessibility of residues in the separate fragments and in the α subunit and α' derivative have given further information on the secondary structure and folding of the fragments. Solvent perturbation studies indicate that only two to three of the seven tyrosyl residues of the α subunit and of the six tyrosyl residues of the α-1 fragment are accessible to solvent. Thus, most of the tyrosyl residues in α and α-1 are located inside the folded protein. This result is consistent with the circular dichroism spectra in the near-ultraviolet (Figure 3) which show that tyrosyl residues are in an ordered structure in both α and α' but possibly in a somewhat different environment in α-1. The single tyrosyl residue in α-2 is accessible to solvent and shows no distinct ellipticity bands. Mixing of the α-1 and α-2 fragments results in a stoichiometric increase in the extinction coefficient ($\Delta\epsilon_{286}$ = 830 M^{-1} cm^{-1}) due to perturbation of tyrosyl residue(s). By use of the value of $\Delta\epsilon_{287}$ = −700 M^{-1} cm^{-1} (Donovan, 1969) for the transfer of a completely buried tyrosine into water, it can be estimated that about 1.2 tyrosines become buried when the fragments are mixed. This value is consistent with the solvent perturbation results which showed three to four tyrosyl residues accessible to solvent in the added sum of the separate fragments and two to three tyrosyl residues accessible to solvent in the α subunit.

Support for Genetic Studies Suggesting Site Interactions. Our demonstration of the presence of two domains in the α subunit supports previous suggestions of possible interaction between sites or domains of the α subunit. Jackson & Yanofsky (1969a,b) demonstrated complementation between mutant α subunits in artificial dimers formed following exposure to 6 M urea. Their finding that most α chains with mutations in the amino-terminal region would complement most α chains with mutations in the carboxy-terminal region implies that these two regions from two different molecules can interact to form an active molecule. In similar complementation studies of β-galactosidase, one of the complementary peptides has been shown to be an independently folding region (or domain) by an immunological study (Celada et al., 1974).

Studies of second-site revertants have also suggested possible site interactions [for a review, see Yanofsky & Crawford (1972)]. The findings that loss of activity in two strains with mutations at residues 211 and 213 was reversed by second-site mutations at residues 175 or 177, respectively, suggest that there is some interaction between a site containing residues 211 and 213 and another site containing residues 175 and 177. Since one of these sites is on α-1 and one is on α-2 in the nicked protein, this region is presumably an important interaction site between the fragments. Furthermore, comparison of primary sequences between tryptophan synthase from *E. coli*, *Sal-*

monella typhimurium, and *Aerobacter aerogenes* (Li & Yanofsky, 1972) showed a region of 100% homology between residue 167 and residue 179. This may be evidence of the importance of retaining the structural integrity of an interaction site. Interestingly, the predictions of Lim include a β-pleated sheet structure for residues 173–179 (Lim, 1974).

Contribution of Results to Understanding of the Structure and Function of Tryptophan Synthase. The structure of the α subunit appears to be composed of two folded regions or domains which are relatively resistant to proteolysis connected by a link containing arginine-188 which is very susceptible to proteolysis by trypsin. This conclusion is based on our previous findings (Miles & Higgins, 1978) that the α-1 and α-2 fragments are the first products of proteolysis of α. The finding that the two proteolytic fragments of the α subunit refold independently suggests that these regions may refold independently in the intact protein [see Högberg-Raibaud & Goldberg (1977a,b) for a related discussion of β_2-subunit folding].

The results of this study and our previous report (Miles & Higgins, 1978) increase our understanding of the arrangement of the α and β_2 subunits and their component domains and interdomain links in the $\alpha_2\beta_2$ complex. Whereas the link between the two α fragments is exposed to proteolysis in the $\alpha_2\beta_2$ complex, the site between the two independently folding domains which is susceptible to proteolysis in the β_2 subunit alone (Högberg-Raibaud & Goldberg, 1977a,b) is protected from proteolysis in the $\alpha_2\beta_2$ complex. Limited proteolysis should be useful for future studies to map the location of residues which are exposed to chemical modification in the $\alpha_2\beta_2$ complex. These studies should further elucidate the topography of the subunits and their domains in the $\alpha_2\beta_2$ complex.

Studies of the functions of the α subunit and its derivatives show that, whereas α, α', and the mixture of the α-1 and α-2 fragments all exhibit enzymatic activity and ability to bind indolepropanol phosphate and β_2 subunit, neither fragment alone has any of these functions. It thus appears that interaction between the two separately refolded fragments is essential for all of these functions. It will be important to determine whether all of the active site of the α subunit is present on one fragment or whether the active site exists in the interaction site between the two fragments.

Although very little is known about the active site of the α subunit, the three sulfhydryl residues, which we now know to be located on the α-1 fragment, have been previously suggested to play an active-site role (Freedberg & Hardman, 1971). Modification of these sulfhydryls results in inactivation and is partially prevented by the substrate indoleglycerol phosphate. Our finding that these three sulfhydryls react much more rapidly with 5,5′-dithiobis(2-nitrobenzoic acid) in the α-1 fragment than in the α subunit or α' suggests that interaction of the two fragments either changes the environment of the sulfhydryl residues within the α-1 fragment or places the sulfhydryl residues in the interaction site between the fragments, thus partially protecting them from solvent. Further work will be necessary to identify active-site residues in the α subunit and to locate them in the folded structure. Use of specific chemical modification of residues on the separate α-1 and α-2 fragments to identify residues essential for catalytic activity may help accomplish this goal.

Acknowledgments

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Supplementary Material Available

Experimental material including amino acid composition of α, α-1, and α-2 (Table I) and sequential Edman degradation of α-1 (Table II) and α-2 (Table III) (5 pages). Ordering information is given on any current masthead page.

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Possibility of Shape Conformers of the Protein Inhibitor of the Cyclic Adenosine Monophosphate Dependent Protein Kinase†

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ABSTRACT: The heat-stable, protein inhibitor of the cyclic adenosine monophosphate (cAMP) dependent protein kinase [Walsh, D. A., Ashby, C. D., Gonzalez, C., Calkins, D., Fischer, E., & Krebs, E. (1971a) *J. Biol. Chem. 246*, 1977–1985] has been purified to homogeneity from rabbit skeletal muscle by preparative electrophoresis. Employing a more sensitive assay system, we detected multiple charged forms of the inhibitor on diethylaminoethyl chromatography; the form that has been further characterizd is the predominant species in skeletal muscle comprising greater than 70% of the total. The apparent molecular weight of the protein inhibitor, as determined by Sephadex G-75 gel exclusion chromatography, is 22 000 in initial cellular extracts and at all stages during the purification prior to the final purification step of preparative gel electrophoresis, after which the homogeneous protein exhibits a molecular weight of 11 000. These two forms are designated I and I′, respectively. The I form migrates with an apparent molecular weight of 10 000 on nondenaturing gel electrophoresis and of 10 500–11 500 on sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis; the I′ form migrates with an apparent molecular weight of 6500–8300 on $NaDodSO_4$ electrophoresis and has a minimum molecular weight of 10 400 by amino acid analysis. Taking into account the anomalous behavior displayed by low molecular weight proteins with the various techniques employed, we suggest that the I and I′ forms of the protein inhibitor may represent shape conformers.

A heat-stable, protein inhibitor of the cAMP-dependent protein kinase, which was partially purified from rabbit skeletal muscle, has been described by Walsh et al. (1971a). This inhibitor had an isoelectric point of 4.1, was precipitated but not inactivated by addition of trichloroacetic acid to 5%, was heat stable to 95 °C, and displayed an apparent molecular weight of ~26 000 on Sephadex G-75. The inhibitor blocked the activity of the cAMP-dependent protein kinase by direct interaction with the free catalytic subunit of the kinase; there was no displacement of the regulatory subunit from the holoenzyme, nor was there formation of a stable inhibitor–holoenzyme complex. These results demonstrated that the inhibitor interacts with the cAMP-dependent protein kinase only after cyclic nucleotide promoted dissociation (Ashby & Walsh, 1972, 1973). The interaction of the inhibitor and catalytic subunit has been shown to be readily reversible (Ashby & Walsh, 1973). Donnelly et al. (1973a,b) have attributed to the inhibitor protein a stimulatory activity toward the cGMP-dependent protein kinase. Subsequent studies by Kuo & Kuo (1976), however, have shown that the cAMP-dependent protein kinase inhibitor and the cGMP-dependent protein kinase modulator are distinct entities. The latter observation is consistent with the protein inhibitor interacting only with the catalytic subunit of the cAMP-dependent protein kinase following dissociation of the holoenzyme and the activation of the cGMP-dependent protein kinase not occurring by a dissociation mechanism (Gill et al., 1976; Lincoln et al., 1977). The modulator has recently been reported to act by interaction with the protein substrate (Shoji et al., 1978).

Since the time of the initial reports (Walsh et al., 1971a; Ashby & Walsh, 1972, 1973), several investigators have described the presence of heat-stable protein kinase inhibitors in various tissues that appear different from that first described. Demaille et al. (1977) purified a heat-stable protein inhibitor from rabbit skeletal muscle of molecular weight 11 000 based on Sephadex G-75 chromatography, $NaDodSO_4$ gel electrophoresis, and amino acid analysis. Weber & Rosen (1977) reported that the inhibitor from bovine cardiac muscle exhibited an apparent molecular weight of ~23 000. Szmigielski et al. (1977) and Costa (1977) have reported the presence of two types of inhibitors of protein kinases in various tissues of molecular weights 24 000 and 14 500. Beale et al. (1977) purified a heat-stable protein kinase inhibitor from rat testis of an apparent molecular weight in the range of 19 000–26 000. A primary purpose of the work described in this paper is to clarify the issue of the molecular size of the inhibitor protein. The heat-stable inhibitor initially described by Walsh et al. (1971a) has been purified to apparent homogeneity by means of preparative gel electrophoresis, and

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