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Identification of Three Sites of Proteolytic Cleavage in the Hinge Region between the Two Domains of the β_2 Subunit of Tryptophan Synthase of *Escherichia coli* or *Salmonella typhimurium*†

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ABSTRACT: The β_2 subunit of tryptophan synthase is composed of two independently folding domains connected by a hinge segment of the polypeptide that is particularly susceptible to limited proteolysis by trypsin [Högberg-Raibaud, A., & Goldberg, M. (1977) *Biochemistry 16*, 4014-4019]. Since tryptic cleavage in the hinge region inactivates the β_2 subunit, the spatial relationship between the two domains is important for enzyme activity. However, it was not previously known whether inactivation results from cleavage of the chain or from the loss of internal fragment(s) subsequent to cleavage at two or more sites. We now report comparative studies of limited proteolysis by three proteinases: trypsin and endoproteinases Lys-C and Arg-C. Our key finding that endoproteinase Arg-C inactivates the β_2 subunit by cleavage at a single site (Arg-275) demonstrates the important role of the hinge peptide for enzymatic activity. We have also identified the sites of cleavage and the time course of proteolysis by trypsin at Arg-275, Lys-283, and Lys-272 and by endoproteinase Lys-C at Lys-283 and Lys-272. Sodium dodecyl sulfate gel electrophoresis, Edman degradation, and carboxypeptidases B and Y have been used to identify the several fragments and peptides produced. Our finding that the β_2 subunit and F1 fragments have a heterogeneous amino terminus (Met-1 or Thr-2) indicates that the amino-terminal methionine is incompletely removed during posttranslational modification. Our results show that Edman degradation can be effectively used with a protein of known sequence to analyze proteolytic digests that have at least four different amino-terminal sequences. Our analysis of the hinge region may lead to further understanding of the importance of this region of the β_2 subunit for activity, allosteric control, and subunit interaction.

Some proteins consist of two independently folding domains interconnected by a hinge peptide that is particularly susceptible to proteolysis (Neurath, 1980; Wetlauffer, 1981; Richardson, 1981). Two such proteins are the tryptophan synthase β_2 subunit (Högberg-Raibaud & Goldberg, 1977a,b) and the tryptophan synthase α subunit (Miles & Higgins, 1978; Higgins et al., 1979; Miles et al., 1982). Although proteolytic cleavage of the α subunit has little effect on enzyme activity, cleavage of the β_2 subunit results in inactivation, loss of cooperative binding of two pyridoxal phosphate molecules to the apo-β_2 subunit, and loss of interaction with the α subunit (Högberg-Raibaud & Goldberg, 1977a,b; Tschopp & Kirschner, 1980; Chaffotte & Goldberg, 1984). These studies and others have provided evidence that the limited flexibility between domains provided by the hinge peptide is often crucial to substrate binding, allosteric control, and assembly of large structures (Richardson, 1981).

Previous studies of the limited proteolysis of the β_2 subunit of tryptophan synthase of *Escherichia coli* and of *Serratia marcescens* have demonstrated the formation of two proteolytic fragments, F1 and F2, with N-terminal sequences starting at Thr-2 and at Val-276 or Ala-284 (Crawford et al., 1978, 1980; Rocha et al., 1979). The observation that the F1 and F2

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fragments appeared on sodium dodecyl sulfate–polyacrylamide gels as doublets in varying proportions after different times of proteolysis (Rocha et al., 1979) suggested that trypsin may cleave at either Arg-275 or Lys-283 depending on the conditions (Crawford et al., 1980).

In order to further map the hinge region of the β_2 subunit and explain the time course and specificity of proteolytic cleavage, we have used three proteolytic enzymes (trypsin, endoproteinase Lys-C, and endoproteinase Arg-C) and have examined the products of proteolysis using Edman degradation and carboxypeptidase digestions. The important finding that endoproteinase Arg-C inactivates the β_2 subunit by cleavage at a single site (Arg-275) demonstrates that chain cleavage and not loss of a fragment of the hinge region is responsible for inactivation.

Experimental Procedures

Endoproteinase Lys-C from *Lysobacter enzymogenes* (Boehringer Mannheim), endoproteinase Arg-C from mouse submaxillary gland (Boehringer Mannheim), carboxypeptidase Y (Sigma), phenylmethanesulfonyl fluoride treated carboxypeptidase B from porcine pancreas (Worthington), and bovine pancreatic trypsin treated with *N*-tosyl-L-phenylalanine chloromethyl ketone (Worthington) were commercial products. The β_2 subunits of tryptophan synthase of *E. coli* and *Salmonella typhimurium* were prepared from the $\alpha_2\beta_2$ complexes and assayed as described (Ahmed et al., 1985; Miles et al., 1986). Since the amino acid sequences of the β chains of *E. coli* and *S. typhimurium* are identical in the hinge region (Crawford et al., 1980), both enzymes have been used interchangeably. Both enzymes yielded the same fragmentation patterns after limited proteolysis by trypsin.

Proteolysis of β_2 Subunit by Trypsin. Proteolysis was performed in 0.05 M sodium *N*,*N*-bis(2-hydroxyethyl)glycine buffer, pH 7.8, containing 2 mM ethylenediaminetetraacetic acid (EDTA), 5 mM β-mercaptoethanol, and 50 μM pyridoxal phosphate at 25 °C. The concentration of β_2 subunit was 5–25 mg/mL and of trypsin was 1.3 μg/mg of β_2 subunit, and the reactions were stopped at the indicated times by adding 10 μg of soybean trypsin inhibitor/μg of trypsin (Högberg-Raibaud & Goldberg, 1977a). The F1 and F2 fragments resulting from tryptic cleavage of the β_2 subunit were isolated by gel filtration in urea (Högberg-Raibaud & Goldberg, 1977a). The F1B and F1A fragments were isolated by this method after trypsin digestions for 5 or 60 min, respectively.

Isolation of an Octapeptide from the Hinge Region after Proteolysis by Trypsin. *S. typhimurium* holo-β_2 subunit (14.4 mg = 320 nmol in 1.5 mL) was treated with trypsin for 60 min as described above, treated with soybean trypsin inhibitor (10 μg/μg of trypsin), and applied to a Sephadex G-25 SF column (0.8 cm × 69 cm). The column was eluted with water at 8 mL/h, collecting 0.3-mL fractions. The main protein peak eluted at the void volume (V_0 = 19.5 mL) and was followed by a second peak (V_e = 28 mL), which was collected and lyophilized. Amino acid analysis of an acid hydrolysate of this material showed that it contained all of the residues of the expected octapeptide Val-276 to Lys-283 of the β_2 subunit plus some additional amino acids probably due to impurities. The material was further purified on a reverse-phase HPLC column (Waters C-18 microbondapack) with a Du Pont 8800 system using a linear gradient of 20–60% acetonitrile in 0.05% trifluoroacetic acid. The major peak (70% of the total material) with retention time = 4.9 min was lyophilized and subjected to Edman degradation.

Proteolysis of β_2 Subunit by Endoproteinase Lys-C. The sample for Edman degradation was prepared by digesting *E. coli* holo-β_2 subunit (2.5 mg = 52 nmol) with endoproteinase Lys-C (0.9 μg = 0.3 unit) in 0.25 mL of 50 mM *N*-ethylmorpholine acetate buffer, pH 7.8, containing 5 mM β-mercaptoethanol and 1 mM EDTA for 5 min at 25 °C. The residual serine deaminase activity after digestion for 5 min was 20%. The digest was treated with 0.25 mL of glacial acetic acid to stop the action of endoproteinase Lys-C, lyophilized, redissolved in water, lyophilized again, and used for Edman degradation. Gel electrophoresis (see below) of an aliquot of this material showed only F1A and F2A bands. Reactions for time course experiments were carried out at 25 °C with 0.4 mg of β_2 subunit and 3 μg of endoproteinase Lys-C in 0.1 mL of 0.05 M sodium phosphate buffer, pH 7.0, containing 5 mM EDTA and 1 mM dithioerythritol.

Protein Concentrations. Protein concentrations were determined from the absorbance with the following specific absorbance values: holo-β_2 subunit, $A^{1\%}_{278nm}$ = 6.5 (Hathaway & Crawford, 1970); F1, $A^{1\%}_{280nm}$ = 0.67; F2, $A^{1\%}_{280nm}$ = 0.45 (Högberg-Raibaud & Goldberg, 1977a).

Carboxypeptidase B Digestion. The *E. coli* F1B fragment (0.8 mg = 30 nmol) was treated with carboxypeptidase B (0.04 mg = 3.2 units) at 25 °C in 0.68 mL of 0.1 M sodium phosphate buffer, pH 7.7, containing 0.1% sodium dodecyl sulfate and 0.1 M NaCl. Aliquots (0.1 mL) were removed at intervals, treated with 15 μL of 1 N HCl, diluted to 0.3 mL with 0.2 M citrate buffer, pH 2.2, and centrifuged. Amino acids in the supernatant were analyzed on a Beckman amino acid analyzer (Beckman 121MB equipped with a 4270 dual-channel integrator) and with norleucine as an internal standard and were corrected for the amounts of amino acids in control mixtures from which carboxypeptidase B or F1B fragment was omitted.

Carboxypeptidase Y Digestion. Digestion was carried out according to Martin et al. (1977). F1A from *E. coli* (1.1 mg = 41 nmol) was incubated at 25 °C with carboxypeptidase Y (0.066 mg = 1.1 nmol) in 0.48 mL of 0.1 M potassium phosphate buffer, pH 7.0, containing 0.1% sodium dodecyl sulfate. Aliquots (0.06 mL) were removed at the indicated times, treated with 1 N HCl (0.006 mL), diluted with 0.2 M citrate buffer, pH 2.2 (0.3 mL), and centrifuged. Amino acids were determined in the supernatant solution as described for carboxypeptidase B digestion. The amino acids found were corrected for the amounts in control mixtures in which F1A or carboxypeptidase Y was omitted.

Gel Electrophoresis. Polyacrylamide gel electrophoresis was performed in the presence of sodium dodecyl sulfate on slabs 3 mm in thickness, according to the method of Laemmli (1970). Gels contained 9.75% w/v acrylamide plus *N*,*N*′-methylenebis(acrylamide) and had 2.63% cross-linker [bis × 100/(acrylamide + bis)]; gels were stained with Coomassie blue. The staining intensity of the bands was determined from densitometric scans of the gels with an LKB No. 2202 ultrascan densitometer.

Edman Degradation. Edman degradation was performed automatically on a modified Beckman sequencer Model 890B (Fairwell & Breuer, 1979, 1980). Phenylthiohydantoin derivatives were identified and determined quantitatively by high-performance liquid chromatography. Since the β_2 subunits treated with proteolytic enzymes contained a heterogeneous amino terminal and two additional amino termini generated by cleavage at Lys-272, Arg-275, or Lys-283, several phenylthiohydantoin derivatives were detected at each cycle and were assigned to known sequences of the β_2 subunit taken from the nucleotide sequences of the *trpB* genes from *E. coli* and from *S. typhimurium* (Crawford et al., 1980). In cases

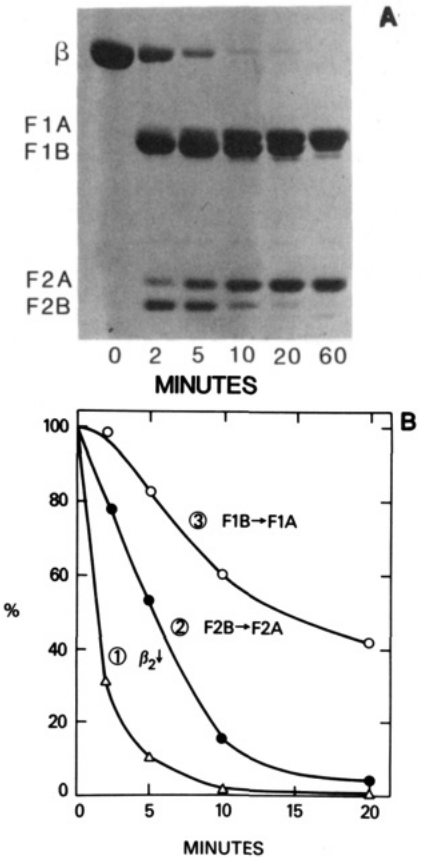

FIGURE 1: Time course of limited proteolysis of the β_2 subunit of tryptophan synthase from *S. typhimurium*. (A) Gel electrophoresis in the presence of sodium dodecyl sulfate of the β chain after the indicated minutes of proteolysis by trypsin (see Experimental Procedures for conditions of proteolysis), gel electrophoresis, and scanning of gels to determine the relative amounts of bands shown in (B). (B) (Δ) Percent of initial intact β chain; (●) percent of F2B band relative to the sum of F2A and F2B bands; (○) percent of F1B band relative to the sum of F1A and F1B bands.

where the same amino acid appears at the same cycle number in two of the sequences being degraded at the same time, the total number of nanomoles recovered has been arbitrarily divided by the number of occurrences, and this number is placed in each sequence within parentheses.

RESULTS

Limited Proteolysis of β_2 Subunit by Trypsin. Figure 1A shows the time course of the tryptic digestion of the β_2 subunit of tryptophan synthase from *S. typhimurium* followed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The two major products of digestion, the F1 and F2 fragments, appear as doublets at intermediate times of digestion, as previously shown for the β_2 subunit from *E. coli* and *S. marcescens* (Rocha et al., 1979). We obtained essentially identical results for the *E. coli* β_2 subunit (data not shown). The faster moving species of each fragment, designated F1B and F2B, predominate after 2 min of digestion, whereas the slower moving species, designated F1A and F2A, predominate after 60 min. Since the results suggest that F1B is converted to F1A and F2B is converted to F2A, the rates of these conversions and of the disappearance of the β_2 subunit were estimated from densitometer scans of the gel (Figure 1B). Our findings that the rate of disappearance of β_2 subunit ($t_{1/2}$ = 1.4 min) is 3.5 times faster than the conversion of F2B to F2A ($t_{1/2}$ = 5 min) and 11 times faster than the conversion of F1B to F1A ($t_{1/2}$ = 15 min) is evidence that at least three cleavage steps occur at these three rates. There are three

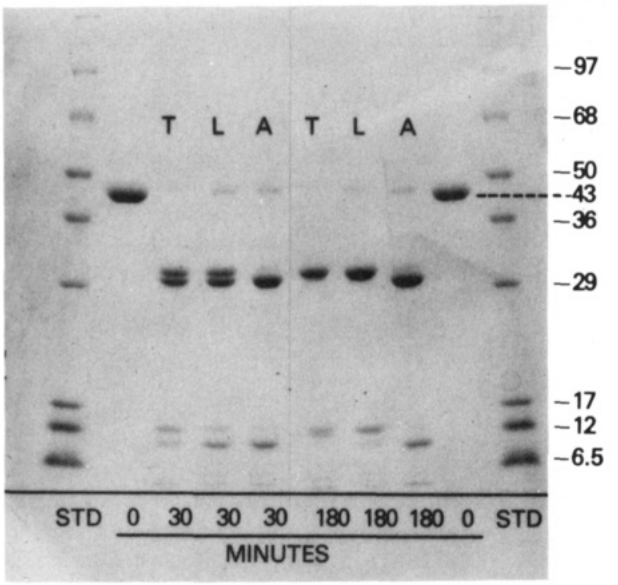

FIGURE 2: Limited proteolysis of the β_2 subunit of tryptophan synthase with trypsin (T), endoproteinase Lys-C (L), and endoproteinase Arg-C (A). Holo-β_2 subunit [4 mg/mL in 0.05 M sodium *N*,*N*-bis(2-hydroxyethyl)glycine buffer, pH 7.8, containing 0.2 mM dithioerythritol and 0.04 mM pyridoxal phosphate] was incubated with either 0.01 mg of trypsin/mL, 0.03 mg of endoproteinase Lys-C/mL, or 0.04 mg of endoproteinase Arg-C/mL at room temperature. Aliquots were removed at 0, 30, and 180 min, boiled with 2 volumes of Laemmli sample buffer, and subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate according to the method of Laemmli (1970). Twenty micrograms of total protein was applied per slot. Molecular weight markers (10^{-3} M_r in parentheses) are glycogen phosphorylase (97), bovine serum albumin (68), indole-3-glycerolphosphate synthase (50), glyceraldehyde-3-phosphate dehydrogenase (36), α subunit of tryptophan synthase (29), myoglobin (17), cytochrome *c* (12), and bovine trypsin inhibitor (6.5).

residues in this region of the β_2 chain potentially susceptible to cleavage by trypsin: Lys-272, Arg-275, and Lys-283 (Crawford et al., 1980) (see Discussion and Figure 5).

Limited Proteolysis of β_2 Subunit by Trypsin, by Endoproteinase Arg-C, and by Endoproteinase Lys-C. In order to simplify the analysis of the sites of cleavage, we have compared proteolysis by trypsin, which cleaves at both lysine and arginine residues, with proteolysis by endoproteinase Lys-C, which cleaves specifically at lysine residues (Jekel et al., 1983; Steffens et al., 1982), and by endoproteinase Arg-C, which cleaves specifically at arginine residues (Schenkein et al., 1977). Figure 2 shows the results of polyacrylamide electrophoresis in the presence of sodium dodecyl sulfate of aliquots of 30-min digestions and of 180-min digestions by each of the three enzymes. The finding that endoproteinase Arg-C produces only one species of each fragment (F1B and F2B) is evidence that these fragments can result from cleavage at Arg-275 (see Discussion).

The action of endoproteinase Lys-C usually produces two polypeptide fragments that move during electrophoresis similar to the final F1A and F2A fragments of trypsin action. Occasionally, under conditions that are not yet well controlled, doublets moving similar to the (F1A + F1B) and (F2A + F2B) fragments of trypsin action are produced as intermediates (Figure 2). This finding is evidence that the slower moving fragments are the products of at least two proteolytic cuts, probably at Lys-272 and Lys-283 (see Discussion and Figure 5). The action of both endoproteinases Arg-C and Lys-C inactivates the β_2 subunit in the same fashion as does trypsin (Högberg-Raibaud & Goldberg, 1977a,b). Moreover, the presence of pyridoxal 5′-phosphate has a dramatic effect on

Table I: Amino Termini of Proteolytic Fragments of β Chain of Tryptophan Synthase from *E. coli* Identified by Simultaneous Sequential Edman Degradation[a]

cycle no.												assignment	
1	2	3	4	5	6	7	8	9	10	11	12	residues	fragment
							Part A						
Met	Thr	Thr	Leu	Leu	Asn	Pro	Tyr	Phe	Gly	Glu	Phe	1–12	F1
7	(2)	4	(14)	9	7	1	10	5	7	9	8		
Thr	Thr	Leu	Leu	Asn	Pro	Tyr	Phe	Gly	Glu	Phe	Gly	2–13	F1
5	(2)	23	(14)	10	3	13	20	(5)	11	14	15		
Val	Gly	Ile	Tyr	Phe	Gly	Met	Lys	Ala	Pro	Met	Met	276–287	F2B
18	13	29	20	20	12	17	17	13	5	9	15		
Ala	Pro	Met	Met	Gln	Thr	Gln	Asp	Gly	Gln	Ile	Glu	284–295	F2A
7	2	7	7	6	2	6	6	(5)	1	5	5		
							Part B						
Met	Thr	Thr	Leu	Leu	Asn	Pro	Tyr	Phe	Gly	Glu	Phe	1–12	F1
13	(21)	5	(22)	18	9	12	12	11	16	13	14		
Thr	Thr	Leu	Leu	Asn	Pro	Tyr	Phe	Gly	Glu	Phe	Gly	2–13	F1
8	(21)	27	(22)	19	24	28	39	(18)	16	28	32		
Val	Gly	Ile	Tyr	Phe	Gly	Met	Lys	Ala	Pro	Met	Met	276–287	F2B
7	15	7	4	9	6	7	7	9	6	3	5		
Ala	Pro	Met	Met	Gln	Thr	Gln	Asp	Gly	Gln	Ile	Glu	284–295	F2A
53	43	37	42	30	20	30	10	(18)	8	27	24		
							Part C						
Val	Gly	Ile	Tyr	Phe	Gly	Met	Lys	(Lys)				276–283	
38	37	28	25	16	9	5	4	(0.4)					
							Part D						
Met	Thr	Thr	Leu	Leu	Asn	Pro	Tyr	Phe	Gly	Glu	Phe	1–12	F1
(3)	2	(9)	7	6	5	8	7	11	6	4			
Thr	Thr	Leu	Leu	Asn	Pro	Tyr	Phe	Gly	Glu	Phe	Gly	2–13	F1
2	(3)	12	(9)	6	6	(13)	(15)	(9)	19	9	9		
His	Gly	Arg	Val	Gly	Ile	Tyr	Phe	Gly	Met	Lys		273–283	
5	4	9	15	10	21	(13)	(15)	(9)	10	3			
Ala	Pro	Met	Met	Gln	Thr	Gln	Asp	Gly	Gln	Ile	Glu	284–295	F2A
22	5	13	16	13	9	14	7	(9)	5	9	8		

[a] (A) Five-minute tryptic digest. (B) Twenty-minute tryptic digest. (C) Peptide isolated from tryptic digest. (D) Five-minute endoproteinase Lys-C digest. Yields of amino acid residues (nmol). Values in parentheses are arbitrarily and evenly distributed for simultaneously appearing amino acid residues (see Experimental Procedures).

the rate and extent of proteolysis. With endoproteinase Lys-C as the protease, apo-β_2 subunit is cleaved about 25-fold more rapidly than holo-β_2 subunit (data not shown). The fragments from both apo- and holo-β_2 subunit are stable toward prolonged proteolysis. Högberg-Raibaud and Goldberg (1977b) report that trypsin degrades F1 and F2 further when pyridoxal 5′-phosphate is removed. Under similar conditions as those of endoproteinase Lys-C, endoproteinase Arg-C cleaves the holo-β_2 subunit about 10-fold more slowly than does endoproteinase Lys-C, and the ratio of cleavage rates of holo-β_2 vs. apo-β_2 subunit is only two (data not shown).

Identification of Amino-Terminal Sequence of Each Fragment and Peptide. Edman degradation of total digests of *E. coli* β_2 subunit treated with trypsin for 5 (Table IA) or 20 min (Table IB) gives four amino acid residues at each cycle, corresponding to Met-1, Thr-2, Val-276, and Ala-284 at cycle 1. The intact β_2 subunit also had Met-1 and Thr-2 at cycle 1 (data not shown). The presence of Met-1 and Thr-2 at cycle 1 is evidence for amino-terminal heterogeneity (see Discussion). The relative proportions of the fragments containing residues 276–397 and residues 284–397 in tryptic digests can be calculated from the yields of Val-276 and Ala-284 in cycle 1 since these residues are known to be stable and to give good yields in Edman degradation. Our finding that the relative proportion of the fragment 284–397 is 28% after 5 min (Table IA), 88% after 20 min (Table IB), and 100% after 60 min (data not shown) is evidence that fragment 284–397 is the F2A band, the F2 band that predominates at the end of digestion.

The stoichiometric relationship between fragment 276–397 and the F2B fragment is demonstrated in Figure 3 by plotting the percent of fragment 276–397 obtained from the end-group

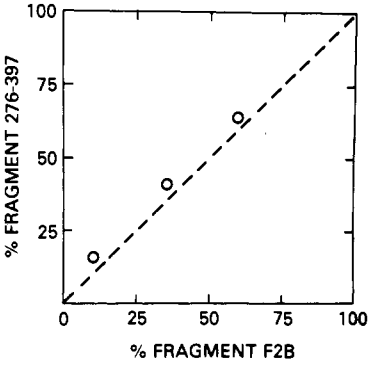

FIGURE 3: Relationship between the proteolytic fragment containing residues 276–397 and the fragment F2B. The percent of fragment 276–397 relative to the sum of fragments 276–397 and 284–397 was calculated from the amounts of Ala and Val in cycle 1 of Edman degradation of three tryptic digests of the β_2 subunit (see Table IA,B). The values are plotted against the percent of fragment F2B relative to the sum of F2B plus F2A obtained from densitometric scanning of gels of the same three samples (see Experimental Procedures. The theoretical relationship for F2B = fragment 276–397 is shown by the dashed line.

analysis (Table I) against the percent of the F2B fragment obtained from gel electrophoresis (Figures 1). Thus, trypsin cleaves the β_2 subunit first at Arg-275 and then at Lys-283. The octapeptide (residues 276–283) that should result from these two cleavage steps was not observed in either of the tryptic digests sequenced in parts A and B of Table I since the sequence of the fragment beginning at Val-276 continued past Lys-283. This small peptide was probably lost during desalting of the sample by gel filtration since we have sub-

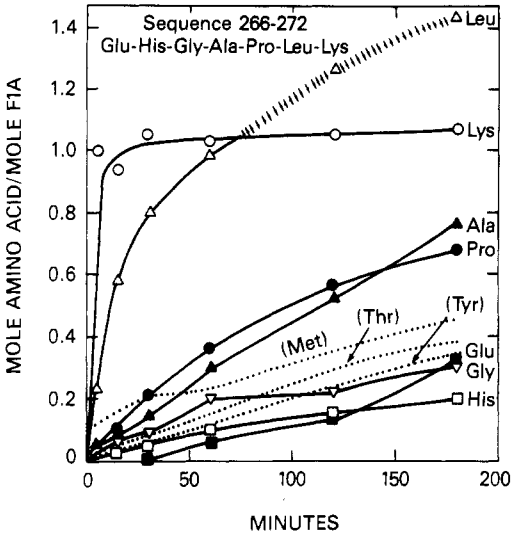

FIGURE 4: Carboxy-terminal amino acids released from the purified F1A fragment by carboxypeptidase Y. The amounts of amino acids released are plotted vs. the time of digestion under conditions described under Experimental Procedures. The amino acids released consistent with the sequence for residues 266–272 are shown with solid lines (—); other amino acids released are shown with dotted lines (···). The amount of Leu released in excess of 1 mol of F1A is shown with a broken line (|||).

sequently isolated this peptide by gel filtration (Table IC).

If endoproteinase Lys-C cleaves the β_2 subunit at both Lys-272 and Lys-283, five fragments should result: F1C (1–283), F1A (1–272), F2C (273–397), F2A (284–397), and the 11-residue peptide (273–283) (see Discussion and Figure 5). The endoproteinase Lys-C digest was carried out in a volatile buffer, which eliminates the gel filtration step and loss of small peptides in preparing the digests for Edman degradation. Table ID demonstrates the presence of F1 fragment(s) with either Met or Thr at the N-terminus, the F2A fragment (284–397), and the undecapeptide. The absence of an alanine residue in cycle 12 demonstrates that all of the fragment with N-terminal His-273 has been cleaved at Lys-283 and that no fragment F2C is present (see Discussion and Figure 5).

Identification of Carboxyl-Terminal Sequence of F1A and F1B. The time course of amino acid release during the limited carboxypeptidase Y digestion of the isolated F1A fragment is given in Figure 4. Our results showing that lysine is released most rapidly and stoichiometrically and that Leu, Pro, Ala, Gly, His, and Glu are released at decreasing rates are consistent with the presence of carboxy-terminal Lys-272 in the carboxy-terminal sequence 266–272 shown in Figure 4. The discrepant findings that more than 1 mol of Leu is released and that three other amino acids (Met, Thr, and Tyr) are released at low rates probably result from the presence of some endoproteolytic activities in the carboxypeptidase Y (Lee & Riordan, 1978).

Since the F1B fragment is thought to result from initial cleavage at Arg-275, it would be expected to have the carboxy-terminal sequence His^{273}-Gly^{274}-Arg^{275}. Neither carboxypeptidase Y nor carboxypeptidase B plus carboxypeptidase A is likely to be useful for establishing this sequence since glycine in the C-terminal position is a very poor substrate for carboxypeptidase A (Ambler, 1967) and glycine as the penultimate carboxyterminal residue is resistant to carboxypeptidase Y digestion (Hayashi, 1977). Treatment of the isolated F1B fragment with carboxypeptidase B resulted in the slow release of Arg (0.8 residue/mol after 3 h) and of Lys (0.2 residue/mol after 3 h). The presence of some lysine is due to the presence of some F1A fragment, which was detected by gel electrophoresis in the presence of sodium dodecyl sulfate.

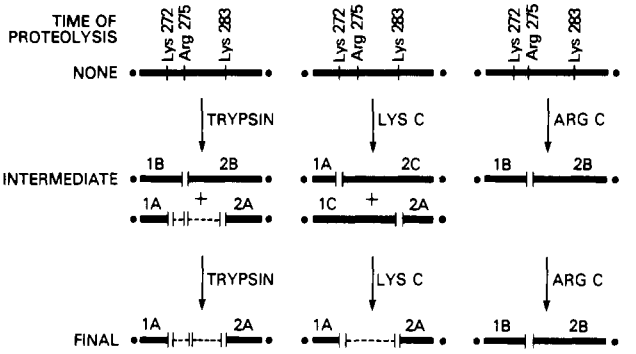

FIGURE 5: Steps in limited proteolysis by trypsin, endoproteinase Lys-C, and endoproteinase Arg-C of the β_2 subunit at a hinge region. The hinge region of the β_2 subunit of tryptophan synthase, a region that is susceptible to limited proteolysis at one or more residues (Lys-272, Arg-275, Lys-283), is shown schematically before proteolysis (·—·). Fragments (·—|) and peptides (|···|) are shown after an intermediate period of proteolysis and after a long period of proteolysis by trypsin, endoproteinase Lys-C, and endoproteinase Arg-C. The fragments produced by tryptic cleavage at Lys-272, Arg-275, and Lys-283 [1A (1–272), 1B (1–275), 2A (283–397), and 2B (276–397)] correspond to the fragments designated F1A, F1B, F2A, and F2B in Figure 1A and identified in Table IA,B and Figures 3 and 4. The identification of the tripeptide (|---|) and octapeptide (|---|) is shown in Table IC and the text. The fragments produced by cleavage at Lys-272 and Lys-283 by endoproteinase Lys-C [1A (1–272), 1C (1–283), 2C (273–397), and 2A (283–397)] correspond to fragments observed as doublets in Figure 2 at 30 min. The undecapeptide and F2A fragment are identified in Table ID. The fragments produced by cleavage at Arg-279 by endoproteinase Arg-C [1B (1–275) and 2B (276–397)] correspond to fragments seen in Figure 2.

Discussion

Sites and Steps of Proteolytic Cleavage. The sequence of the "hinge region" of the β_2 subunit, the sites of cleavage, and the fragments and peptides that we have identified are shown in Figure 5. The products of tryptic cleavage are F2A (284–397) and F2B (276–397), which are identified by Edman degradation (Table IA,B and Figure 3), F1B (1–275) identified by its C-terminal Arg with carboxypeptidase B, F1A (1–272) identified by its C-terminal sequence with carboxypeptidase Y (Figure 4), the octapeptide (276–283), which was isolated and identified by Edman degradation (Table IC), and the tripeptide (273–275). Although the tripeptide has not been isolated and conclusively identified, its amino acids (His-Gly-Arg) have been detected in cycles 1–3 of Edman degradation of a 1.5-h trypsin digest of β_2 subunit (data not shown). Since this digest contained about 10 residues at each cycle, rather extensive digestion has occurred during this period. The initial appearance of F1B (1–275) and F2B (276–397) in the tryptic digestion is consistent with a first step of tryptic cleavage at Arg-275. This conclusion is supported by the finding that cleavage by endoproteinase Arg-C produces fragments that appear on gels to be identical with the F1B and F2B fragments produced by trypsin (Figure 2). Trypsin may react more selectively with arginine than with lysine in proteins since the K_{cat}/K_m is more than 10 times greater for a model arginine compound than for a model lysine compound (Craik et al., 1985). The next two steps of tryptic cleavage occur at quite different rates (Figure 1B): cleavage of F2B (276–397) at Lys-283 to produce F2A (284–397) and the peptide 276–283 and cleavage of F1B (1–275) at Lys-272 to produce F1A (1–272) and the peptide 273–275.

Edman degradation of a total endoproteinase Lys-C digest (Table ID) demonstrates the products of endoproteinase Lys-C digestion: F1A (1–272), an undecapeptide (273–283), and F2A (284–397), which result from cleavage at Lys-283 and

Lys-272. These two cleavage reactions can occur stepwise as shown in Figure 5 and by the two protein bands at early times in Figure 2. In this case, the putative larger product of initial cleavage at Lys-283, F1C = 1–283, appears to have the same mobility as F1B (1–275). Similarly, F2C (273–397) appears to have the same mobility as F2B (276–397).

Anomalous Mobility of Fragments on Gel Electrophoresis. Although the mobility of polypeptides in gel electrophoresis in the presence of sodium dodecyl sulfate is usually inversely proportional to the molecular weight (Shirahama et al., 1974; Takagi et al., 1975), we find that the proteolytic fragments of the β_2 subunit migrate anomalously (Figure 1). That is, the larger of the two F1 fragments, F1B (1–275), migrates more rapidly than F1A (1–272), and the larger of the two F2 fragments, F2B (276–397), migrates more rapidly than F2A (284–397). Anomalies of this sort have been found previously (Kubo et al., 1982; Tung & Knight, 1971) and are thought to be due to differences in the net negative charge per unit length of these polypeptides. Although the empirically established relationship between mobility on gels in the presence of sodium dodecyl sulfate and molecular weight is based on the assumption that polypeptides bind a constant amount of sodium dodecyl sulfate (1.4 g/g) and thus have a fixed constant negative net charge per unit length of protein polypeptide, Takagi et al. (1975) report that the maximum amount of binding can vary from 1.2 to 1.5 g/g.

A chemical modification reaction (maleylation of amino groups) that increases the net negative charge of proteins around neutral pH values decreases the mobility and thus increases the apparent molecular weight (Tung & Knight, 1971). Application of this finding to the interpretation of our results suggests that the F1A fragment may have a lower mobility than the larger F1B fragment because the F1A fragment has two more net negative charges due to removal of His-273 and Arg-275. Similarly, the F2A fragment may have a lower mobility than the larger F2B fragment because the F2A fragment has one more net negative charge due to removal of Lys-283. Our results suggest that care should be taken in interpreting the molecular weights of proteins by sodium dodecyl sulfate gel electrophoresis.

Amino Terminus of β_2 Subunit. Edman degradation of the nicked β_2 subunit (Table IA,B,D) and of the intact β_2 subunit (data not shown) shows the presence of two amino-terminal sequences starting at Met-1 and Thr-2 of the sequence deduced from the DNA sequence (Crawford et al., 1980). About one-fifth of each amino terminus is Met-1 and four-fifths is Thr-2. These results indicate that the removal of amino-terminal methionine by an aminopeptidase (Watson, 1976) is incomplete in this strain of *E. coli* (*trpRΔtrpLD102*/F′*ΔtrpLD102*). In contrast, previous studies of β_2 subunit isolated from *E. coli trpA2*/F′*colVBtrpA2* showed that threonine was the only amino terminus (Crawford et al., 1978; Higgins et al., 1980). The strains differ in two ways: (1) the strain used in these studies produces higher levels of the β_2 subunit than the strain used previously and (2) the strain used here produces the $\alpha_2\beta_2$ complex, whereas the strain used before produced only β_2 subunit. Our results suggest that the amount of aminopeptidase activity in the highly overproducing strain is inadequate to process all of the β_2 subunit produced. Alternatively, formation of the $\alpha_2\beta_2$ complex in this strain might partially prevent this processing step.

An earlier step in processing of bacterial polypeptide chains is the removal of the formyl group from *N*-formylmethionine by a deformylase (Watson, 1976). *N*-Formylmethionine has been found at some of the amino termini of the β_2 subunit from *E. coli* strain PW11, harboring a multicopy plasmid containing the trp operon (Tsunasawa et al., 1983). Edman degradation of the intact β_2 subunit cannot be used to detect the presence or absence of *N*-formylmethionine, since a blocked amino terminus is not cleaved. However, the presence or absence of a blocked amino terminus can be deduced from sequence analysis of nicked β_2 subunits. Edman degradation of a nicked β_2 subunit containing a free amino terminus should give equal amounts of F1 fragments with amino-terminal Met-1 or Thr-2 and of F2 fragments with amino-terminal Val-276 and Ala-284, whereas degradation of a nicked β_2 subunit containing a blocked amino terminus should give no residues from the F1 fragment but the expected yield of residues from the N-terminal sequence of the F2 fragment. Since each of the three nicked β_2 subunits in Table IA,B,D gives an approximately equal yield of residues from the N-terminal sequences of the F1 and F2 fragments, we conclude that these β_2 subunits are not blocked at the amino terminus. Our results also show that Edman degradation can be effectively used with a protein of known sequence to analyze proteolytic digests that have four different amino-terminal sequences.

Importance of Hinge Region. Our results have helped to map the hinge region between the two domains of the β_2 subunit and to show the relative susceptibility of three sites to proteolysis: Lys-272, Arg-275, and Lys-283. Cleavage at Lys-272 has not been previously recognized. Since limited proteolysis of the β_2 subunit destroys its catalytic activity, its ability to bind two pyridoxal phosphate molecules cooperatively, and its ability to interact with the α subunit (Högberg-Raibaud & Goldberg, 1977a,b; Tschopp & Kirschner, 1980; Chaffotte & Goldberg, 1984), the intact hinge region may be necessary for holding the two domains in the conformation necessary for these three properties. The LexA repressor, which is inactivated by either proteolytic cleavage of or deletions within the connector between two domains (Little & Hill, 1985), represents an interesting analogy. Because cleavage of a single peptide bond by endoproteinase Arg-C also leads to inactivation of the β_2 subunit, loss of the 11 amino acids between Lys-272 and Ala-284 cannot be the primary cause of inactivation. Insertion of a single glycine residue between Lys-272 and His-273 via oligonucleotide–directed mutagenesis does not affect the enzyme activity of the mutant protein (Ch. Binkert and K. Kirschner, unpublished experiments). This result suggests that generation of charged C- and N-termini at the proteolytic cleavage sites, rather than relief of tension by proteolysis of a tautly strung polypeptide connector, is responsible for inactivating the β protomer. The direct or indirect involvement of this hinge region in interaction with the α subunit is also supported by the finding that the β_2 subunit is not susceptible to proteolysis by trypsin in the $\alpha_2\beta_2$ complex (Miles & Higgins, 1978).

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Registry No. Tryptophan synthase, 9014-52-2; trypsin, 9002-07-7; endoproteinase Lys-C, 72561-05-8; endoproteinase Arg-C, 82047-85-6.

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Soluble Fibrin–Fibrinogen Complexes as Intermediates in Fibrin Gel Formation

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ABSTRACT: Oligomer formation in fibrinogen solutions following addition of thrombin was studied by addition of thrombin inhibitor at various times subsequent to thrombin, followed by size-exclusion chromatography (SEC) on a high-performance SEC column capable of resolving species of molecular weights $\leq 10^6$. Peaks corresponding to species with 1, 2, 3, and 4 or more times the molecular weight of fibrinogen were detected and quantified via nonlinear least-squares curve-fitting procedures. The evolution of each of these peaks with time is well accounted for by a kinetic model in which the predominant component of each oligomeric molecular weight species is a linear complex of fibrinogen and fibrin. The observed predominance of trimeric over dimeric oligomers even at short times suggests that the thrombin-catalyzed release of the two A fibrinopeptides from a single molecule of fibrinogen is highly cooperative.

Numerous studies of the thrombin-catalyzed conversion of fibrinogen to fibrin and the concomitant formation of fibrin gels have been carried out with the intention of elucidating the mechanism of fibrin gel formation [for a recent review, see Scheraga (1983)]. Time-dependent studies carried out under quasi-physiological conditions, as monitored by a variety of techniques (Ferry & Morrison, 1947; Shinowara, 1966; Brass et al., 1976; J. Wilf and A. P. Minton, unpublished results) have shown two distinctly different types of kinetics, depending upon the concentration of thrombin which is added to initiate the reaction. In the presence of high concentrations of thrombin, the characteristic time for appearance of a gel following addition of thrombin decreases monotonically with increasing fibrinogen concentration. In contrast, at low concentrations of thrombin, the characteristic time goes through a minimum at some intermediate value of fibrinogen concentration and increases significantly at higher fibrinogen concentrations. Although this biphasic behavior has been

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