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LOCATION OF THE REACTIVE SULFHYDRYL RESIDUES IN THE PRIMARY SEQUENCE OF THE β_2 SUBUNIT OF TRYPTOPHAN SYNTHASE OF ESCHERICHIA COLI

Edith Wilson Miles and William Higgins*

Laboratory of Biochemical Pharmacology
National Institute of Arthritis, Metabolism, and Digestive Diseases
National Institutes of Health, Bethesda, Maryland 20205

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SUMMARY Two of the 5 sulfhydryl residues of the β_2 subunit of tryptophan synthase have previously been shown to react with N-ethylmaleimide and to have active site roles. We now show that the single sulfhydryl which reacts with N-ethylmaleimide in the presence of pyridoxal phosphate is cysteine-170. The essential sulfhydryl which reacts with N-ethylmaleimide or with 2-nitro-5-thiocyanobenzoic acid after removal of pyridoxal phosphate is cysteine-230. The affinity reagent, bromoacetylpyridoxamine phosphate, reacts variably with cysteine-62 or with cysteine-230.

We have previously studied the reactivity of active site sulfhydryl residues in the β_2 subunit of tryptophan synthase with N-ethylmaleimide (NEM)[1] (1, 2), 2-nitro-5-thiocyanobenzoic acid (2), and an affinity reagent, bromoacetylpyridoxamine phosphate (2-4). The recent determination of the primary sequence of the β_2 subunit by DNA sequence analysis (5) now enables us to locate these reactive sulfhydryl residues in the primary sequence and to clarify the relationships between these residues and the active site. The sulfhydryl which reacts with NEM in the presence of pyridoxal phosphate (SH-II) is cysteine-170. The essential sulfhydryl which reacts with NEM or with 2-nitro-5-thiocyanobenzoic acid in the absence of pyridoxal phosphate (SH-I) is cysteine-230. Bromoacetylpyridoxamine phosphate reacts with cysteine-230 under some conditions (3) and with cysteine-62 under other conditions (4).

MATERIALS AND METHODS

Materials — N-[Ethyl-2-^{3}H]maleimide ([^{3}H]NEM) (10,400 cpm/nmol) and N-[ethyl-1-^{14}C]maleimide ([^{14}C]NEM) (2,000 cpm/nmol) were from New England Nuclear. 2-Nitro-5-thio[^{14}C]cyanobenzoic acid (8,900 cpm/nmol) was prepared by the method of Degani and Patchornik (6). [^{3}H]Pyridoxal phosphate (10^3 cpm/nmol) was synthesized by a modification (7) of the original (8) method.

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* Present address, Centre de Recherche Merrell International, 16, rue d'Ankara, 67084 Strasbourg Cedex, France.

[1] Abbreviation used: NEM, N-ethylmaleimide.

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Methods — Limited proteolysis, isolation of F_1 and F_2 fragments, cyanogen bromide cleavage, purification of peptides, and amino acid analysis[2] were as described (4). Tryptic hydrolysis using TPCK-trypsin (Worthington) at 50 μg/ml for 3-5 hours at 37° was performed either in 0.1 M N-ethylmorpholine acetate buffer, pH 9.0, or in 0.025 M potassium phosphate, pH 7.8, containing 2 M urea. Paper chromatography and electrophoresis were by the method of Bennett (4) as described previously (10). The apo β_2 subunit was prepared from the $\alpha_2\beta_2$ complex of tryptophan synthase as described (11, 12) and modified as described in Table I.

TABLE I

Preparations of modified β_2 subunit *

Preparation	Designation	Residues modified				Fig.
		SH-II [a]	SH-I [b]	PLP site [c]	Other 3 SH residues [d]	
1	[^{3}H]PLP-[^{14}C]NEM-I	NEM	[^{14}C]NEM	[^{3}H]PLP		1, 2B
2	[^{14}C]NEM-SH-1-5 [d]	[^{14}C]NEM	[^{14}C]NEM	-	[^{14}C]NEM	2A
3	[^{14}C]NEM-I	NEM	[^{14}C]NEM	-	NEM	2C
4	[^{14}C]CN-[^{3}H]NEM-II	[^{3}H]NEM	[^{14}C]NTCB	-	NEM	2D, E

* Abbreviations used in table: NEM, N-ethylmaleimide; PLP, pyridoxal phosphate; NTCB, 2-nitro-5-thiocyanobenzoic acid; CN-, enzyme modified by conversion of -SH to -SCN by reaction with NTCB.

[a] Holo β_2 subunit (about 5 mg in 1 ml 0.1 M potassium phosphate buffer, pH 7.8, containing 1 mM EDTA, 0.45 M $(NH_4)_2SO_4$, and 0.1 mM pyridoxal phosphate), was treated with 0.25 mM NEM, [^{14}C]NEM, or [^{3}H]NEM for 30 minutes at 22° followed by addition of 2.5 mM β-mercaptoethanol and 0.01 M NH_2OH.

[b] Apo β_2 subunit labeled at SH-II was transferred into the buffer used in [a] above minus pyridoxal phosphate by dialysis or gel filtration and then treated for 30 minutes at 22° with 0.25 mM [^{14}C]NEM or for 60 minutes at 22° with 0.7 mM [^{14}C]NTCB.

[c] Apo β_2 subunit labeled at SH-II and SH-I with NEM and dialyzed against 0.05 M sodium N,N-bis-(2-hydroxyethyl)glycine buffer, pH 7.8, containing 1 mM EDTA and 1 mM dithiothreitol, was treated with a stoichiometric amount of [^{3}H]pyridoxal phosphate and then with 1 mM $NaBH_4$.

[d] Modified or unmodified enzyme in 0.1 M potassium phosphate, pH 7.8, containing 1 mM EDTA and 8 M urea, was treated for 30 minutes at 22° with 0.25 mM NEM or [^{14}C]NEM.

RESULTS AND DISCUSSION

The cyanogen bromide digest of the F_1 fragment which had been isolated from the modified enzyme ([^{3}H]PLP-[^{14}C]NEM-I [Table I, Preparation 1]) was fractionated by gel filtration on Sephadex G-25 (Fig. 1). Most of the radio-

[2] We are grateful to Mrs. Barbara F. Torain, Laboratory of Chemistry, NIAMDD, National Institutes of Health, for the amino acid analyses.

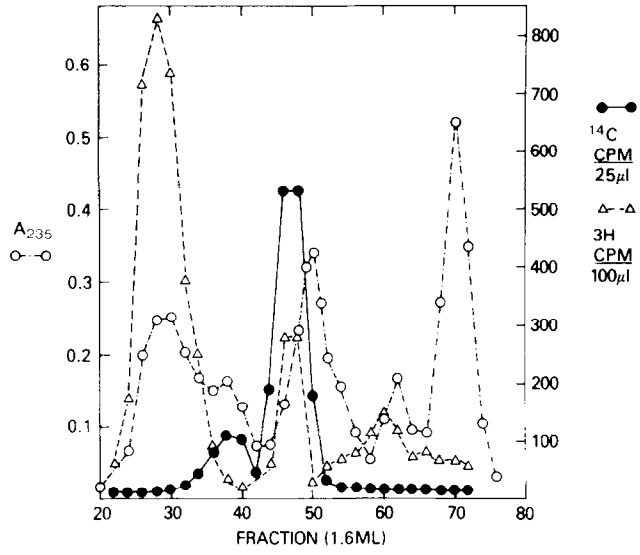

FIG. 1. Gel filtration of a cyanogen bromide digest of [^{3}H]PLP-[^{14}C]NEM-I-F_1 on Sephadex G-25 (fine). [^{3}H]PLP-[^{14}C]NEM-I β_2 (Table I, Preparation 1) was subjected to limited proteolysis by trypsin; the isolated F_1 fragment was digested with cyanogen bromide. The lyophilized digest in 1 ml 0.1 N acetic acid was applied to a column of Sephadex G-25 (fine) (1.5 cm x 88 cm) and eluted with 0.1 N acetic acid, collecting 1.6 ml fractions. Absorbance at 235 nm (θ) and radioactivity of ^{14}C (●) and ^{3}H (Δ) were determined.

activity from the [^{3}H]pyridoxal phosphate eluted in a single large fragment centered at Fraction 28, which we previously (4) identified as Residues 23-100. Since the radioactivity from the [^{14}C]NEM was almost entirely in a smaller fragment eluted in Fractions 46-48, the cysteine labeled by [^{14}C]NEM cannot be cysteine-62.

A tryptic digest of the β_2 subunit which had been uniformly labeled with [^{14}C]NEM (Table I, Preparation 2) was fractionated by gel filtration on Biogel P-4 into radioactive peaks 1-5 (Fig. 2A). These 5 peaks should contain the five cysteine-containing peptides which would be expected from tryptic digestion of the β chain (5) (Table II). The largest of these peptides (Residues 223-272) is the only one to contain a methionine (Residue 240) and should be cleaved by cyanogen bromide to yield a cysteine containing peptide (Residues 223-240). Since cyanogen bromide treatment of the combined fractions from peak 1, Fig. 2A, gave a radioactive fragment eluting at Fraction 38 (Fig. 2B), peak 1 presumably contains Residues 223-272. A radioactive fragment eluting near Fraction 38 (Fig. 2B) was also obtained by gel filtration of a tryptic digest of the cyanogen bromide fragment labeled at SH-I (see Fig. 1) or by gel filtration of a cyanogen bromide digest of the single tryptic peptide isolated from β_2 labeled at SH-I (Fig. 2C). Since the amino acid composition of the

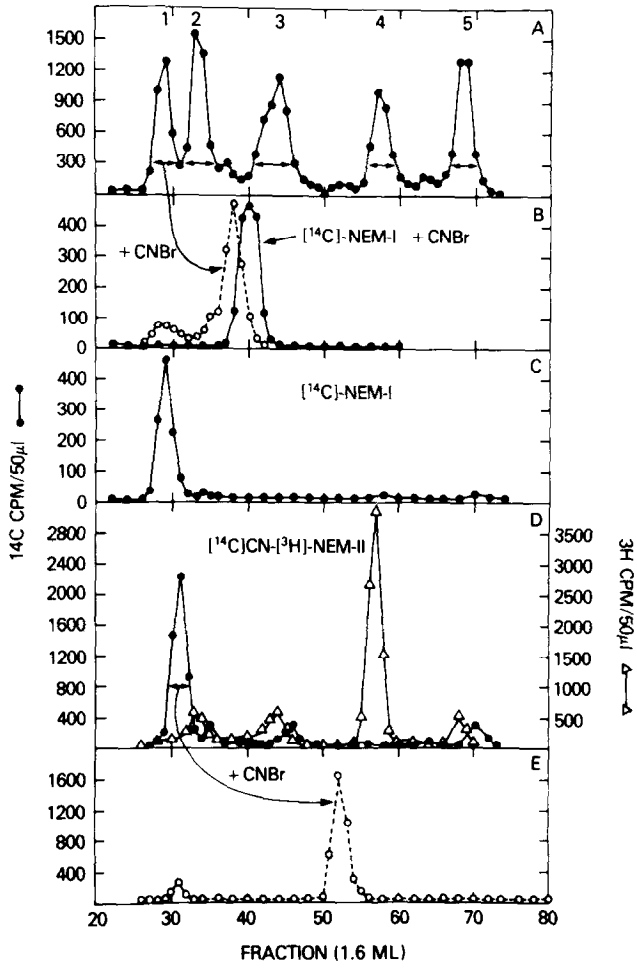

FIG. 2. Gel filtration of tryptic peptides on Biogel P-4. Tryptic digests of modified β_2 subunit (A, C, D) or cyanogen bromide fragment (B, E) were applied to a Biogel P-4 column (1.5 cm x 90 cm) and eluted with 9% formic acid collecting 1.6 ml fractions. Radioactivity due to ^{14}C (O or ●) or ^{3}H (Δ) was determined on 50 µl aliquots. (A) Tryptic digest of β_2 labeled at all sulfhydryl residues with [^{14}C]NEM (Table I, Preparation 2). (B) Tryptic digest of the main ^{14}C component in the cyanogen bromide digest of [^{3}H]PLP-[^{14}C]NEM-I-F_1 (Fractions 44-50, Fig. 1), ●——●; a cyanogen bromide digest of combined Fractions 2-30 from Fig. 2A, O——O. (C) Tryptic digest of [^{14}C]NEM-I (Table I, Preparation 3). (D) Tryptic digest of [^{14}C]CN-[^{3}H]NEM-II (Table I, Preparation 4). (E) Cyanogen bromide digest of combined Fractions 30-32 from Fig. 2D, O——O.

cyanogen bromide fragment of the tryptic peptide labeled at SH-I (see Table III: NEM-I + CNBr) is closer to that expected for Residues 220-240 than for Residues 223-240, arginine-222 is probably resistant to tryptic cleavage; thus peak 1 actually contains Residues 220-272. The important conclusion from these results is that SH-I is cysteine-230.

Gel filtration of a tryptic digest of ([^{14}C]CN-[^{3}H]NEM-II β_2 [Table I, Preparation 4]) gave a major ^{3}H-labeled peak at the position of tryptic peak

TABLE II

Amino acid sequence of tryptic peptides containing cysteine residues predicted from the complete sequence of the β_2 subunit as determined by DNA sequence (5)

Residues	Sequence
223-272 or 220-272	220 225 230 235 (Glu-Gly-Arg) Leu Pro Asp Ala Val Ile Ala Cys Val Gly Gly Gly Ser 240 245 250 Asn Ala Ile Gly Met Phe Ala Asp Phe Ile Asn Glu Thr Asn Val Gly Leu 255 260 265 Ile Gly Val Glu Pro Gly Gly His Gly Ile Glu Thr Gly Glu His Gly Ala 270 Pro Leu Lys
338-360	340 345 350 Thr Leu Cys Leu His Glu Gly Ile Ile Pro Ala Leu Glu Ser Ser His Ala 355 360 Leu Ala His Ala Leu Lys
62- 76	65 70 75 Cys Gln Asn Ile Thr Ala Gly Thr Asn Thr Thr Leu Tyr Leu Lys
168-175	170 175 Asp Ala Cys Asn Glu Ala Leu Arg
130-131	130 Cys Arg

4 and a major ^{14}C-labeled peak at Fractions 30-32 near the position of tryptic peak 1 (Fig. 2D). Cyanogen bromide treatment of the tryptic peak containing the [^{14}C]CN- peptide, yielded a ^{14}C-labeled peptide (Fig. 2E) which was smaller than the cyanogen bromide cleavage product of NEM-I (Fig. 2B) and had the correct amino acid composition for Residues 230-240 (Table III). This peptide would result from cleavage of the polypeptide chain at two points: after methionine-240 and before cysteine-230. Cleavage probably occurred at cyano-cysteine-230 in our experiment during dialysis for 16 hours at 22° against 0.1 M potassium phosphate buffer, pH 7.8, containing 8 M urea, prior to trypsin digestion. Degani and Patchornik (13) and Jacobsen et al. (14) have reported that cyano-proteins are cleaved under alkaline conditions and in the presence of denaturing agents. Treatment of [^{14}C]CN β_2 under these conditions yielded a ^{14}C-labeled fragment with a molecular weight of 19,500,[3] consistent with cleavage at cysteine-230. The finding that the ^{14}C-labeled tryptic peak eluted about 2 fractions later (Fig. 2D) than the [^{14}C]NEM labeled peak 1 (Figs. 2A and 2C) is consistent with prior cleavage at cysteine-230 to yield a 43-residue tryptic peptide containing Residues 230-272.

[3] Unpublished experiments of W. Higgins using gel filtration on a calibrated Sephadex G-75 column.

TABLE III

Amino acid compositions of isolated peptides and expected sequences [a]

Amino acid	Peptide											
	CN-1 + CNBr	230-240 [a]	NEM-I + CNBr	220-240 [a]	223-240 [a]	NEM-2	338-360 [a]	NEM-4	168-175 [a]	NEM-5	130-131 [a]	
	number of residues											
Cys	0.9 [b]	1	0.6 [b]	1	1	1.2 [b]	1	1.1 [b]	1	0.8 [b]	1	
Asp(n)	1.1	1	2.1	2	2	0.3		2.2	2	0.1		
Thr	- [c]		-			0.8	1	-		-		
Ser	1.0	1	1.1	1	1	2.1	2	-		0.2		
Glu(n)	0.2		1.0	1		2.3	2	1.4	1	0.2		
Pro	-		1.0	1	1	0.9	1	-		-		
Gly	3.8	4	5.0	5	4	1.5	1	0.4		0.3		
Ala	1.1	1	2.9	3	3	4.0	4	2.3	2	-		
Val	0.8	1	1.4	2	2	-		-		-		
Met	-	1 [d]	0.3 [d]	1 [d]	1 [d]	-		-		0.2		
Ile	0.9	1	1.8	2	2	0.6	2	-		-		
Leu	0.2		1.2	1	1	4.9	5	1.0	1	-		
Tyr	-		-			-		-		-		
Phe	-		-			-		-		-		
His	-		-			2.7	3	-		-		
Lys	-		-			1.0	1	-		-		
Arg	-		0.9	1		-		1.0	1	1.0	1	

[a] Calculated compositions for peptides shown (see Table II for sequences).

[b] Estimated from radioactivity.

[c] "-" indicates less than 0.1 residue/mole or undetected.

[d] Homoserine found or expected after CNBr digestion.

[^{14}C]NEM-labeled tryptic peptides in peaks 2-5 (Fig. 2A) were further purified by paper electrophoresis or paper chromatography, eluted, and acid-hydrolyzed for amino acid analysis.[2] The labeled peptides from peaks 2, 4, and 5 had amino acid compositions (Table III) close to those expected for Residues 338-360, 168-175, and 130-131, respectively. Thus SH-II which is contained in a peak eluting in the position of peak 4 is cysteine-170. Although the NEM-labeled peptide in peak 3 was not sufficiently pure for a good analysis, this peptide was isolated and sequenced in our previous report (4).

Interrelations between Cysteine Residues 62, 170, 230, and the Active Site of the β_2 Subunit — The results of this study clearly identify cysteine-170 as the sulfhydryl which is reactive with NEM in the presence of pyridoxal phosphate (SH-II) and cysteine-230 as the sulfhydryl which reacts with NEM after removal of pyridoxal phosphate (SH-I). Cysteine-230 is further shown to

react with 2-nitro-5-thiocyanobenzoic acid to yield a cyano-protein, thus confirming our previous conclusion (2) that the same sulfhydryl (SH-I) reacted with both 2-nitro-5-thiocyanobenzoic acid and NEM in the apo enzyme. That study also concluded that SH-I was essential for catalytic activity since modification by a small, uncharged cyano group destroyed catalytic activity.

Cysteine-230 also appears to react with bromoacetylpyridoxamine phosphate under the conditions of our first report (3). The amino acid composition of the tryptic peptide containing the affinity label resembles that expected for Residues 220-272 and notably contains a methionine.[4] Cyanogen bromide digestion of the typtic peptide yielded 2 fragments which were separated by gel filtration on Biogel P-6. The amino acid compositions of these fragments are consistent with sequences now known (5) for Residues 220-240 and 241-272.[5] In our more recent report (4) the primary site of reaction of bromoacetylpyridoxamine phosphate was shown to be cysteine-62. These results suggest that bromoacetylpyridoxamine phosphate may react with either cysteine-62 or cysteine-230 depending on the conditions. Alterations in the specificity of affinity reagents depending on the conditions have been noted previously (15).

One difference between the conditions of modification in the two studies (3, 4) was that the holo enzyme was first treated with NEM in the sequence study (4). Since bromoacetylpyridoxamine phosphate can react with either Residue 62 or 230, these residues may be located close together and close to pyridoxal phosphate in the active site of the folded β_2 subunit. It is hoped that further studies with cross-linking reagents using the peptide isolation methods developed in this and our previous report (4) will enable us to map the distances between cysteine-62, -170, and -230 in the folded protein structure.

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[4] This peptide was later found to be partially impure.

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