Vol. 82, No. 1, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 15, 1978 Pages 265-272

STUDIES OF THE FUNCTION AND LOCATION OF TWO CYSTEINES IN THE β_2 SUBUNIT OF TRYPTOPHAN SYNTHASE

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Received March 27,1978

SUMMARY: Our findings that the apo β_2 subunit of tryptophan synthase of *Escherichia coli* is inactivated by the modification of one sulfhydryl residue per monomer by nitrothiocyanobenzoic acid and is reactivated by removal of the CN group indicate that the reactive sulfhydryl residue (SH-I) is essential for catalytic activity. SH-I is shown to be the same residue which was previously found to react with bromoacetylpyridoxamine phosphate and different from a sulfhydryl (SH-II) which reacts with N-ethylmaleimide in the presence of pyridoxal phosphate. The results of partial tryptic digestions of β_2 subunit labeled selectively at SH-I or SH-II show that both sulfhydryl residues are located in the F_1 fragment which also contains the pyridoxal phosphate binding site.

Our previous study of the reaction of the β_2 subunit of tryptophan synthase with 5,5'-dithio-bis(2-dinitrobenzoic acid) (DTNB) [1/] and N-ethylmaleimide (NEM) concluded that two reactive sulfhydryl residues may be in the active center of each β monomer (1). Since modification of one sulfhydryl residue by NEM in the presence of pyridoxal phosphate (PLP) resulted in loss of some, but not all, of the activities of the enzyme, this residue, designated SH-II, cannot be essential for catalytic activity but may serve some other active center role (1). Since modification of a second residue by DTNB or NEM in the absence of PLP results in the loss of all activities, this residue, designated SH-I, may be essential for catalytic activity (1). Nitrothiocyanobenzoic acid (NTCB) is a useful reagent for determining whether a sulfhydryl residue serves an essential catalytic function (2, 3). It introduces a small, uncharged CN group which is less likely to inhibit an enzyme by steric effects or by conformational distortion than do the bulky or charged groups introduced by some other reagents such as DTNB or NEM (2, 3). Our new finding that NTCB reacts specificially with one

[1/] Abbreviations used: BAPMP, bromoacetylpyridoxamine phosphate; NTCB, 2-nitro-5-thiocyanobenzoic acid; NEM, N-ethylmaleimide; DTNB, 5,5'-dithio-bis(2-dinitrobenzoic acid); TNB, thionitrobenzoic acid; PLP, pyridoxal phosphate; DEPC, diethylpyrocarbonate; bicine, (N,N-bis(2-hydroxyethyl)glycine.

0006-291X/78/0821-0265$01.00/0

sulfhydryl group per apo β monomer and that activity is regenerated when the CN group is removed, demonstrates that this sulfhydryl is essential for catalytic activity.

The sulfhydryl modified is shown to be the same residue which was previously designed SH-I and the same sulfhydryl which is modified by the affinity reagent, bromoacetylpyridoxamine phosphate (BAPMP) (4). Thus, the sulfhydryl which is close to the pyridoxal phosphate binding site and is protected from modification by DTNB, NEM, or BAPMP by pyridoxal phosphate, is also essential for catalytic activity. Finally, using the method of Högberg-Raibaud and Goldberg for the tryptic cleavage of the β_2 subunit into two fragments (5, 6), we find that both SH-I labeled with BAPMP or NTCB and SH-II labeled with NEM are located in F_1, the fragment which has previously been shown to contain the pyridoxal phosphate binding site (5, 6). Goldberg has found that each of the two sulfhydryl groups accessible to labeling by NEM is located in F_1. [2/]

MATERIALS AND METHODS

Crystalline apo β_2 subunit of tryptophan synthase was purified from *E. coli* strain *trpA2*/F'*trpA2* by the method of Miles and Moriguchi (7) or from *E. coli* strain *trpR*⁻Δ*trpED102*/F'Δ*trpED102* by the method of Högberg-Raibaud and Goldberg (6). NTCB and [^{14}C]NTCB (7.4 mCi/mmole) were prepared by the method of Degani and Patchornik (2). [^{14}C]BAPMP (1.4 mCi/mmole) was prepared as described (4). NEM and [1-^{14}C]NEM (13 mCi/mmole) were purchased from Schwarz/Mann. [^{3}H]Pyridoxal phosphate (53 mCi/mmole) was synthesized by a modification (8) of the original method (9). Sodium dodecyl sulfate polyacrylamide gels were run according to Laemmli (10). Protein was stained with Coomassie blue, destained, and extracted with H_2O_2 (11). The serine deaminase activity of the β_2 subunit was measured spectrophotometrically (12).

RESULTS AND DISCUSSION

Sulfhydryl Modification of the β_2 Subunit. The results of treatment of the β_2 subunit with various sulfhydryl reagents are shown in Fig. 1 and Table I and in a scheme shown in Fig. 2. As shown previously (1), DTNB and NEM react with two sulfhydryl groups per monomer of apo β_2 subunit and one sulfhydryl group per monomer of holo β_2 subunit (Fig. 1 and Table I, experiments 1, 2, 9, and 10). As described in the introduction, the sulfhydryl reacting with NEM or DTNB in the presence of PLP has been designated as SH-II, whereas the second sulfhydryl,

2/ Personal communication from Michel Goldberg, June 1977.

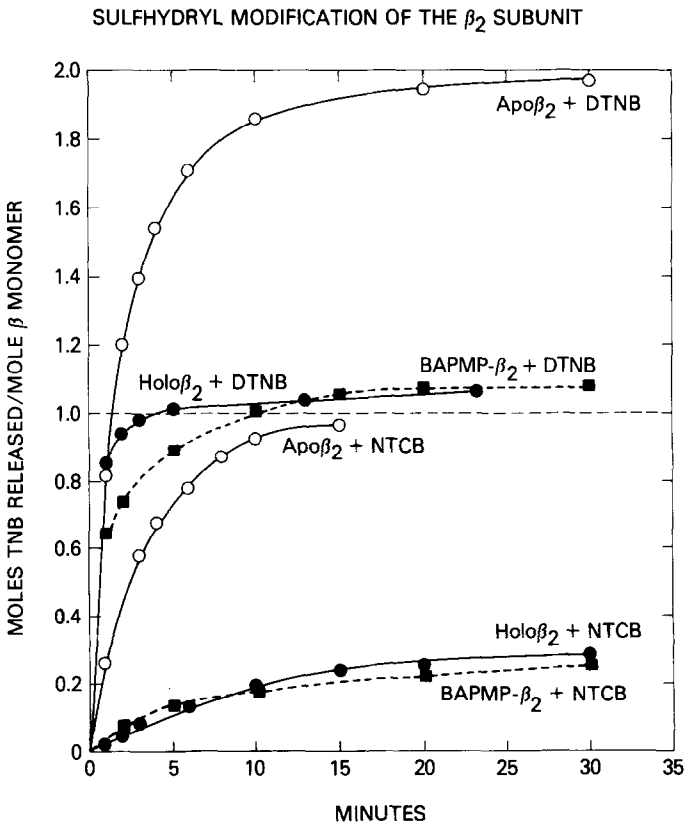

FIG. 1. Reaction of the β_2 subunit with DTNB and NTCB. Apo β_2 subunit [1.2 mg/ml in 0.1 M potassium phosphate, pH 7.8, containing 0.1 mM EDTA and 0.45 M $(NH_4)_2SO_4$ (buffer A)] was treated with 0.1 mM DTNB or NTCB (O) at 25°. Holo β_2 subunit (prepared by addition of 0.1 mM pyridoxal phosphate to the reaction mixture) was treated under the same conditions (O). BAPMP-β_2, prepared by treating apo β_2 subunit (1.3 mg/ml in 0.05 M sodium bicine, pH 8.0), with 0.7 mM [^{14}C]BAPMP for 60 minutes at 37° and subsequent dialysis against buffer A for 20 hours at 4°, was treated with 0.1 mM NTCB or 0.1 mM DTNB (■ and dashed lines). Moles of TNB released per mole β monomer was calculated from the absorbance at 412 nm (1).

which also reacts with NEM or DTNB in the apoenzyme but is shielded by PLP from reaction with NEM or DTNB in the holoenzyme, has been designated SH-I (see Fig. 2). In contrast, NTCB modifies only one sulfhydryl group per monomer of apo β_2 subunit and is thus more specific than DTNB or NEM. The greater specificity of NTCB is surprising and may be related to the fact that it is a less reactive sulfhydryl reagent than is DTNB (3). The finding that NTCB causes complete inactivation of the apo β_2 subunit by modification of one SH per monomer with a

TABLE I. *Modification and inactivation of the apo β_2 subunit and its derivatives by sulfhydryl reagents*

Experiment	Form of enzyme [a]	Reagent	moles incorporated / mole β monomer	Serine deaminase activity %	Reference
1	apo β_2	DTNB	1.8, 2.0 [b]	0	(1), Fig. 1
2	holo β_2	DTNB	1.0, 1.1 [b]	10 [d]	(1), Fig. 1
3	apo β_2	NTCB	1.0 [b]	0	Fig. 1
4	holo β_2	NTCB	0.3 [b]	60	Fig. 1
5	apo β_2	BAPMP	0.8 [c]	0	(4)
6	holo β_2	BAPMP	0.0 [c]	100	(4)
7	BAPMP-β_2	NTCB	0.3 [b]	0	Fig. 1
8	BAPMP-β_2	DTNB	1.1 [b]	0	Fig. 1
9	apo β_2	NEM	1.8 [c]	0	(1)
10	holo β_2	NEM	1.0 [c]	130 [d]	(1)
11	NEM-apo β_2 [a]	BAPMP	0.5 [c]	3	[e]
12	NEM-apo β_2 [a]	NTCB	0.8 [b]	3	[f]

[a] Enzymes were prepared as described in Fig. 1. NEM-apo β_2 (labeled solely at SH-II) was prepared by treatment of holo β_2 with NEM (experiment 10 above) followed by removal of the pyridoxal phosphate by treatment with 1 mM NH_2OH followed by dialysis for 16 hours against 0.05 M Na bicine, pH 7.8.

[b] Measured by the release of TNB at 412 nm after 30 minutes at 25° (1).

[c] Measured by the incorporation of radioactivity (1, 4).

[d] Modification of holo β_2 subunit by DTNB causes loss of serine deaminase activity whereas modification by NEM under the same conditions leads to increased serine deaminase activity but loss of tryptophan synthase activity (1). This difference is probably due to the larger, charged group introduced by DTNB.

[e] NEM-apo β_2 (2.7 mg/ml in 0.05 M Na bicine, pH 7.8) was treated with 0.15 mM NTCB for 30 minutes at 25°.

[f] NEM-apo β_2 (2.7 mg/ml in 0.05 M Na bicine, pH 7.8) was treated with 1.4 mM [^{14}C]BAPMP for 30 minutes at 37°.

small, uncharged CN group suggests that this SH is essential for activity (Table I, experiment 3). Removal of the CN substituent by treatment for 25 minutes with a final concentration of 0.14 M β-mercaptoethanol completely restored activity. Since pyridoxal phosphate largely prevents modification of apo β_2 subunit by either BAPMP (4) or by NTCB (Fig. 1 and Table I, experiment 4) and

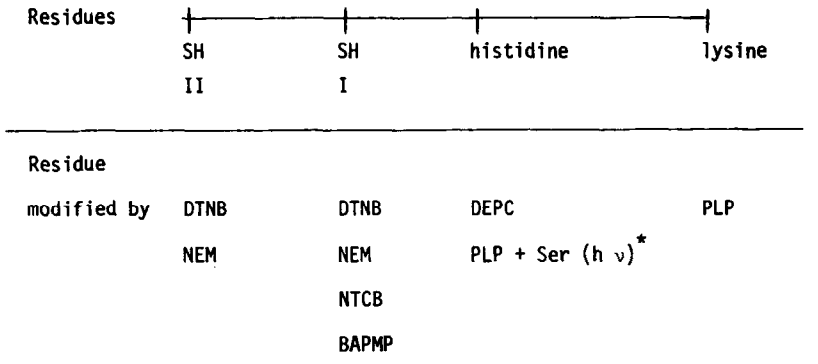

FIG. 2. Scheme showing several active site residues of the β_2 subunit. PLP + Ser (h ν) indicates pyridoxal phosphate-sensitized photooxidation (14, 15). (The scheme is not intended to represent the order of these residues on the polypeptide chain.)

also prevents the modification of the sulfhydryl designated SH-I by NEM and DTNB, we concluded tentatively that BAPMP and NTCB both react with SH-I. The finding that BAPMP-modified β_2 does not react appreciably with NTCB (Fig. 1 and Table I, experiment 7) is consistent with the conclusion that both reagents modify the same residue. The final proof that both reagents react with SH-I was obtained by preparing β_2 subunit labeled at SH-II only with NEM, removing the PLP, and showing that this NEM-apo β_2 reacts with and is inactivated by NTCB and BAPMP (Table I, experiments 11 and 12).

These results are all consistent with the model shown in Fig. 2, which shows that SH-I reacts with DTNB, NEM, NTCB, and BAPMP, whereas SH-II reacts primarily with DTNB and NEM. The essential sulfhydryl residue (SH-I) is close to the pyridoxal phosphate binding site since it is protected by pyridoxal phosphate from modification by DTNB, NEM, NTCB, and BAPMP and since it can be labeled stoichiometrically in the apoenzyme by BAPMP which acts as a pyridoxal affinity reagent (4). Fig. 2 shows two other groups known to be in the active site of the β_2 subunit: the lysyl residue, which forms a Schiff base with pyridoxal phosphate (13) and an essential histidyl residue which is located in the pyridoxyl tryptic peptide (14, 15).

Limited Proteolysis of the β_2 Subunit. In order to obtain further infor-

TABLE II. *Location of modified cysteine residues in the fragment of the β_2 subunit which binds pyridoxal phosphate* [a]

Experiment	Reagent used to modify SH-II	Reagent used to modify SH-I	PLP form	β monomer control	F_1 fragment	F_2 fragment
				cpm	*% of control*	*cpm*
1	-		[^{3}H]-reduced [b]	781	96	5
2	[^{14}C]NEM [c]	-	PLP	430	54	2
3	-	[^{14}C]BAPMP [d]	BAPMP	228	69	12
4	-	[^{14}C]NTCB [e]	PLP	168	72	4
5	[^{14}C]NEM	[^{14}C]BAPMP [f]	BAPMP	994	99	2

[a] Modified β_2 subunit (1 mg/ml in 0.05 M sodium buffer, pH 7.8) was treated with bovine pancreatic trypsin treated with N-tosyl-L-phenylalanine chloromethylketone (Worthington) at 5 μg/ml for 30 minutes at 20° and then with soy bean trypsin inhibitor (Sigma) at 25 μg/ml. Aliquots containing 10 μg of modified β_2 subunit before or after trypsin treatment were run on sodium dodecyl sulfate polyacrylamide gels according to Laemmli (10) except that β-mercaptoethanol was omitted from the sample buffer in experiment 4. After staining and destaining, bands corresponding to β monomer and F_1 and F_2 fragments (see Fig. 3) were cut out, extracted with H_2O_2 (11), and counted. The total counts in the β monomer from untreated enzymes are shown; the counts in the F_1 and F_2 fragments are shown as a percent of the counts in the control β monomer band.

[b] β_2 subunit to which [^{3}H]pyridoxal phosphate was covalently attached by reduction with $NaBH_4$ was prepared by the method of Högberg and Goldberg (5).

[c] Prepared by the method of Miles (1) from holo β_2 subunit.

[d] Prepared by the method of Higgins and Miles (4) from apo β_2 subunit.

[e] Prepared from apo β_2 subunit as shown in Fig. 1 and then reconstituted with pyridoxal phosphate.

[f] Prepared by resolution of [^{14}C]NEM β_2 subunit (experiment 2), followed by treatment with [^{14}C]BAPMP as described in Table I, experiment 11.

mation about the structural relationships of SH-I, SH-II, and the pyridoxyl peptide in the β chain, we have cleaved variously modified β_2 subunit with trypsin by the method of Högberg-Raibaud and Goldberg (5, 6). The β_2 subunit is cleaved into two large nonoverlapping polypeptide fragments, F_1 and F_2, which can be separated on sodium dodecyl sulfate acrylamide gels (Fig. 3). We have confirmed that the F_1 fragment contains all of the label from [^{3}H]pyridoxal phosphate which has been covalently attached to the β_2 subunit by $NaBH_4$ reduction (Table II) (5). In addition, we find that SH-II labeled with [^{14}C]NEM and SH-I labeled with [^{14}C]BAPMP or [^{14}C]NTCB are located in the F_1 fragment. The

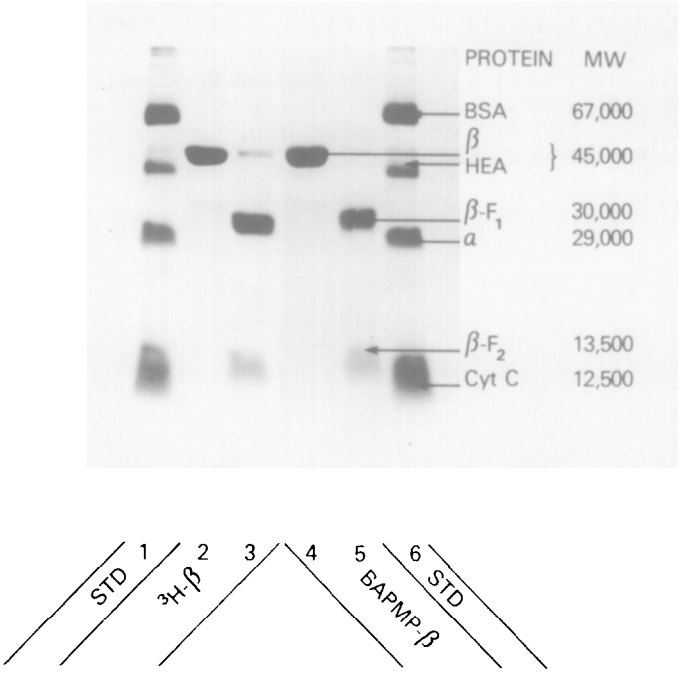

FIG. 3. Sodium dodecyl sulfate polyacrylamide gels of modified β_2 subunit before and after trypsin treatment. β_2 subunit modified as described in Table II (experiments 1 and 3) was treated with trypsin and run on gels as described in Table II. Slots 2 and 3: [^{3}H]-reduced β_2 subunit before (2) and after (3) trypsin treatment. Slots 4 and 5: BAPMP-β_2 subunits before (4) and after (5) trypsin treatment. The molecular weights of the fragments F_1 and F_2 were determined from the migration of protein standards in slots 1 and 6: bovine serum albumin (BSA) (67,000); hen egg albumin (HEA) (45,000); α subunit of tryptophan synthase (α) (29,000); and cytochrome c (cyt c) (12,500).

F_1 fragment thus contains all of the active site residues shown in Fig. 2 and could be useful for future studies of the active site region of the β_2 subunit.

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