EVIDENCE THAT THE ESSENTIAL, PHOTOSENSITIVE HISTIDYL RESIDUE IN THE β_2 SUBUNIT OF TRYPTOPHAN SYNTHETASE IS IN THE PYRIDOXYL PEPTIDE

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The β_2 subunit of tryptophan synthetase of *Escherichia coli* is photoinactivated in the presence of pyridoxal 5'-phosphate and L-serine as a result of the destruction of one histidyl residue per chain (1). Two tryptic peptides are found in much lower amounts in the photoinactivated enzyme than in the control enzyme. These peptides have been identified from their amino acid composition as the 9 or 10 residue peptides which terminate with the lysyl residue which forms a Schiff base with pyridoxal 5'-phosphate. These peptides contain two histidyl residues, one of which appears to be photosensitive. Thus pyridoxal 5'-phosphate sensitizes the photooxidation of a nearby, essential histidyl residue.

We have previously reported that the β_2 subunit of tryptophan synthetase is inactivated by illumination in the presence of its cofactor, pyridoxal 5'-phosphate, and a substrate, L-serine (1). Photoinactivation appears to be due to the destruction of one histidyl residue since analysis of the photoinactivated enzyme showed the loss of about 0.8 histidyl residue per chain and the loss of no tyrosyl, tryptophanyl, methionyl, or cysteinyl residues (1). This investigation attempts to determine whether a specific histidyl residue is photooxidized and to determine the location of this histidyl residue by comparing tryptic digests of photoinactivated (modified) and control β_2 subunit.

MATERIALS AND METHODS

The β_2 subunit was purified by the method of Adachi and Miles [1/] (see reference 1). Aliquots of the β_2 subunit at 2 mg/ml in 0.1 M potassium phosphate

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1/ I wish to thank Dr. Osao Adachi for preparing the β_2 subunit.

buffer, pH 7.8, containing 0.02 mM pyridoxal 5'-phosphate and 0.1 M L-serine were illuminated for 8 minutes as described in reference 1; the modified enzyme had a serine deaminase activity which was 6% of that of the control. Modified and control enzymes were precipitated with cold TCA (5% w/v final concentration) and were washed 3 times each with cold 5% w/v TCA, acetone, and ether to remove buffer, pyridoxal 5'phosphate, and water. The air dried proteins were oxidized with performic acid (2), diluted, and lyophilized. The protein (27 mg) was dissolved in 0.4 ml 1 N NH_4OH at 37°. NH_4HCO_3 (0.4 ml of a 1 M solution) and 30 µl of 1% TPCK-trypsin (Worthington) in 1 mM HCl were added. The mixture was stirred at 37° for 6 hours. Additional trypsin (30 µl) was added after 1 hour. These conditions of tryptic digestion are somewhat more vigorous than those used by Crawford and coworkers (3) for the isolation of the pyridoxyl peptide (1% w/w trypsin for 1.5 hours at 37°).

Peptide maps were made by the method of Bennett (4) using chromatography in 1-butanol : glacial acetic acid : H_2O (4 : 1 : 5) organic phase for 24 hours in the first dimension and electrophoresis for 1 hour at 2,500 v in pyridine : H_2O : glacial acetic acid (2.7 : 350 : 1) at pH 5.4 in the second dimension. Ninhydrin positive spots were detected after dipping chromatograms in 0.1% ninhydrin in acetone followed by overnight drying at room temperature in the dark. Ninhydrin spots were faded by a dip in acetone containing 1% concentrated HCl. Chromatograms were then sprayed with Pauly reagent (4). Spots to be analyzed were cut out, washed with acetone, and eluted with 2 ml 6 N HCl. The eluted peptides were hydrolyzed in evacuated tubes at 110° for 24 hours, taken to dryness, and were then analyzed on a Beckman Model No. 120C amino acid analyzer using the accelerated (120 ml/hour) system (5). [2/]

The histidyl content of fractions from Sephadex G-25 chromatography of tryptic digests was determined by the diazonium tetrazole method (6). Aliquots

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[2/] These analyses were kindly performed by Mr. George Poy, Arthritis and Rheumatism Branch, NIAMDD, NIH.

(0.1 ml) of fractions in 0.1 M NH_4HCO_3 were taken to dryness in a desiccator *in vacuo*. Each residue was dissolved in 0.7 ml of 1 M $KHCO_3$, pH 8.3, and treated at pH 8.8 with 2-0.2 ml aliquots of diazonium tetrazole reagent at 10 minute intervals. Absorbance at 480 nm and 550 nm was determined after 90 minutes.

RESULTS AND DISCUSSION

Tryptic digests of control and photooxidized β_2 subunit show closely similar elution patterns on Sephadex G-25 as judged by absorbance at 280 nm (Fig. 1A). However, one fraction (tubes 43-47) of the digest of the photo-

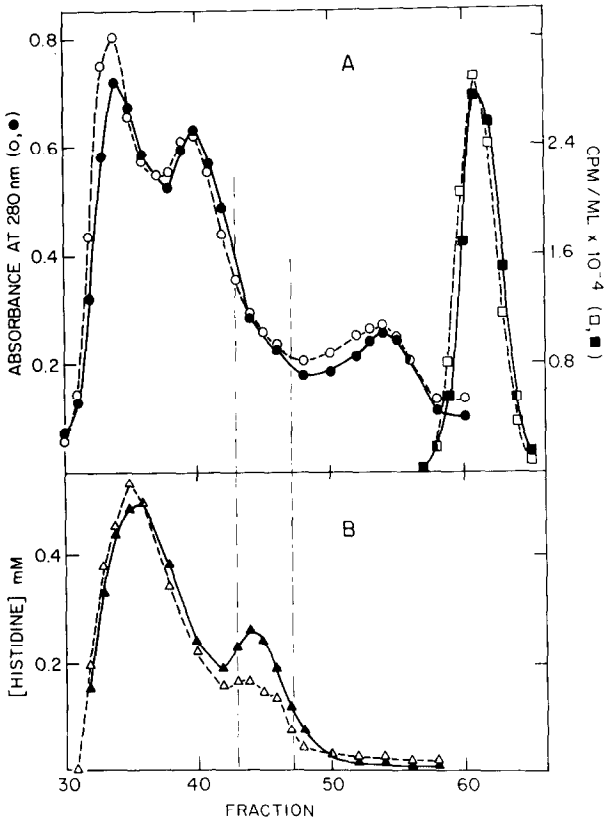

FIG. 1. *Chromatography of tryptic digests on Sephadex G-25.* A tryptic digest from 22 mg of β_2 subunit in 0.7 ml containing 0.5 μCi of [U-^{14}C]L-serine as a marker was applied to a 0.9 x 120 cm column of Sephadex G-25 superfine in 0.1 M NH_4HCO_3. One ml fractions were collected at 4 ml/hour.

A. *Absorbance at 280 nm from digests of control (●) and photooxidized (○) β_2 subunit.* CPM/ml due to [U-^{14}C]L-serine in the control (■) and photooxidized (□) β_2 subunit digests.

B. *Histidyl content of digests of control (▲) and photooxidized (△) β_2 subunit digests.* See *Materials and Methods*.

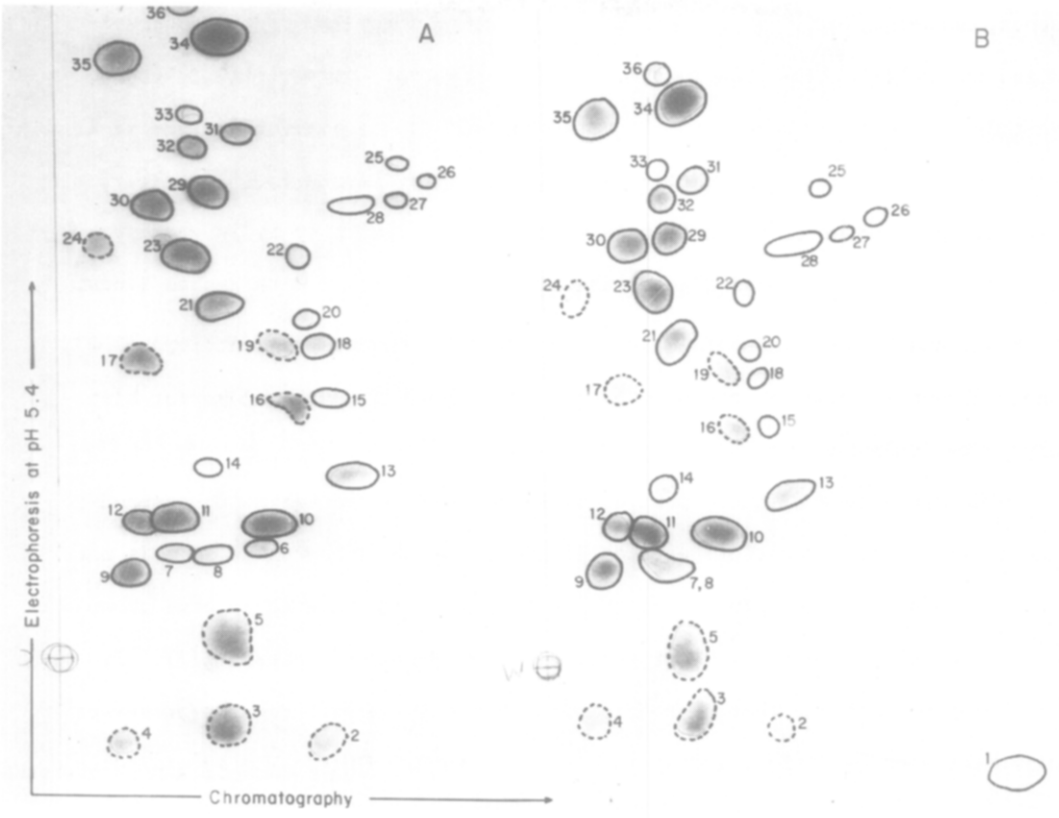

FIG. 2. *Peptide maps of tryptic digests of control (A) and photooxidized (B) β_2 subunit.* Aliquots of tryptic digests from 4.3 mg of β_2 subunit were subjected to peptide mapping (see *Materials and Methods*). Spots indicated by dashed lines were also observed on peptide maps (not shown) of aliquots of combined fractions 43-47 from Sephadex G-25 chromatography of the main part of the same tryptic digest (see Fig. 1). Spot 1 also appeared on the peptide map of the control digest but fell outside of the area photographed in Fig. 1A.

oxidized enzyme showed a distinctly lower content of histidyl residues (Fig. 1B) than did the corresponding fractions of the digest of the control enzyme. The difference in histidyl content in these fractions between the control and photooxidized enzyme digests was 340 nmoles, which is equivalent to 0.7 mole per mole of β monomer. This difference accounts for most of the loss of histidine in the photoinactivated enzyme which was about 0.8 histidyl residue per β monomer (1).

Peptide maps of aliquots of the tryptic digests are shown in Fig. 2. Two

spots, numbers 17 and 24, are distinctly fainter in the map of the photooxidized enzyme (Fig. 2B) than in the map of the control enzyme (Fig. 2A). [3] Peptide maps were also made on aliquots (25%) of the combined fractions 43-47 from the Sephadex G-25 chromatograms of the tryptic digests (not shown). The several spots observed in these maps were identified with spots in corresponding locations of the total digests and are shown in Fig. 2 with dashed lines. Spots 17 and 24 were identified as major spots in the maps of fractions 43-47. Spots 17 and 24 on both sets of maps gave strong positive reactions for histidine with Pauly reagent.

The appearance of a new peptide in the photooxidized enzyme corresponding to the disappearance of peptides 17 and 24 was not observed. This is not surprising since model studies on the photooxidation of histidine have shown that numerous products may be produced, some of which are unstable (7). It is therefore probable that photooxidation of the β_2 subunit results in several different species differing only in histidine photoproduct. The several different tryptic peptides arising from this region might be present in too low an amount to detect.

Peptides 17 and 24 from the maps of fractions 43-47 were eluted, hydrolyzed, and submitted to amino acid analysis as described in *Materials and Methods*. The results are shown in Tables I and II which also give the amino acid composition of peptide T-23 which has been isolated by Crawford and coworkers (3). It is evident that the analyses for the peptides from the digest of the control enzyme (17C and 24C) and from the modified enzyme (17M and 24M) both have compositions closely similar to that reported for T-23. The main difference between peptides 17 and 24 is that the glutamic acid content of 17 is considerably less than that of 24 and less than one residue per mole

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[3] Other spots also appear fainter in Fig. 2B, but this is probably due to unequal ninhydrin development or photography of the two maps, since controls in which spots number 16 were eluted from both maps and subjected to hydrolysis and amino acid analysis gave the same number of nmoles of each amino acid present (data not shown).

TABLE I

AMINO ACID ANALYSIS OF PEPTIDES 24 AND T-23

	24C [a]		24M [b]		T-23
	nmoles	moles per chain	nmoles	moles per chain	moles per chain
Lys	6.1	[1.0]	2.1	[1.0]	1
His	9.1	1.5	3.7	1.8	2
Asp	6.2	1.0	2.3	1.1	1
Glu	7.0	1.1	2.4	1.1	1
Gly	13.5	2.2	4.3	2.0	2
Ala	7.0	1.1	2.1	1.0	1
Leu	13.7	2.2	3.3	1.6	2

[a] Peptide 24 from the tryptic digest of the control β_2 subunit.
[b] Peptide 24 from the tryptic digest of the photooxidized β_2 subunit.

when the data are normalized to lysine equals 1. We presently have no explanation for the origin of a peptide lacking one glutamic acid or glutamine residue. The total recovery of peptides 17C + 24C was 15.4 nmoles from 62 nmoles of β chain. The recovery of the corresponding peptides 17M + 24M was 5.7 nmoles. Thus it appears that about 73% of the peptides 17 and 24 are destroyed during irradiation. Under these conditions about 94% of the activity of the enzyme was destroyed. The additional activity loss may be due to other nonspecific, secondary effects of irradiation as discussed in reference 1.

The results show that most of the histidine which is destroyed during photoinactivation of the β_2 subunit can be accounted for by the decrease of

TABLE II

AMINO ACID ANALYSIS OF PEPTIDES 17 AND T-23

	17C [a]		17M [b]		T-23
	nmoles	moles per chain	nmoles	moles per chain	moles per chain
Lys	9.3	[1.0]	3.6	[1.0]	1
His	19.1	2.0	7.0	2.0	2
Asp	8.4	0.9	3.4	1.0	1
Glu	2.9	0.3	1.6	0.5	1
Gly	21.0	2.3	7.9	2.2	2
Ala	10.4	1.1	4.0	1.0	1
Leu	19.0	2.0	7.2	2.0	2

[a] Peptide 17 from the tryptic digest of control β_2 subunit.

[b] Peptide 17 from the tryptic digest of photooxidized β_2 subunit.

TABLE III

AMINO ACID SEQUENCE OF TRYPTIC PEPTIDES FROM

E. COLI TRYPTOPHAN SYNTHETASE β_2 SUBUNIT (3)

Pyx-1

Glu-Asp-Leu-Leu-His-Gly-Gly-Ala-His-Lys*-Thr-Asn-Gln-Val-Leu-Gly-Gln-Ala-Leu-Leu-Ala-Lys

T-23

Glu-Asp-Leu-Leu-His-Gly-Gly-Ala-His-Lys

* The lysyl residue indicated by the asterisk is present as the εN-phosphopyridoxyl lysyl derivative after the β_2 subunit is reduced by $NaBH_4$ in the presence of pyridoxal 5'-phosphate.

histidine in a certain fraction of tryptic peptides from chromatography on Sephadex G-25. Only 2 of the 8 peptides in this fraction, peptides 17 and 24, showed a strong Pauly reaction for histidine and a significant decrease in ninhydrin color in the photoinactivated enzyme. The amino acid contents of these peptides are close to that of peptide T-23, which was isolated by Crawford and coworkers (3). The published sequence of peptide T-23 corresponds to the 10 residues on the N-terminal end of a larger 22 residue peptide, Pxy-1, which was isolated from a tryptic digest of β_2 subunit to which pyridoxal 5'-phosphate had been covalently bound with NaB^3H_4 (3) (Table III).

Thus it appears that the histidyl residue in the β_2 subunit which is susceptible to photoinactivation is one of the two histidyl residues located in the 10 residue peptide on the N-terminal side of the lysyl residue which normally forms a Schiff base linkage with pyridoxal 5'-phosphate. This conclusion is further supported by our findings (not shown) that peptides 17 and 24 are not present in peptide maps of tryptic digests of $NaBH_4$ reduced control enzyme. Thus photooxidation results in the destruction of a specific histidyl residue which is in close proximity to the enzyme-bound pyridoxal 5'-phosphate.

These data indicate that even though the pyridoxal 5'-phosphate Schiff base linkage with the ε-amino group of the protein lysine is broken when the Schiff base with serine is formed, either the histidyl residue in the peptide chain adjacent to this lysyl residue, or the one 4 residues away, remains in close proximity to the serine - pyridoxal 5'-phosphate Schiff base complex. We have previously suggested (1) that this histidyl residue serves to remove the α-proton of L-serine, a step which is rate-limiting in the conversion of L-serine to pyruvate by the β_2 subunit (8).

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