Structural and Functional Roles of the Cysteine Residues in the \( \alpha \) Subunit of the \textit{Escherichia coli} Tryptophan Synthetase

III. STUDIES WITH THE BIFUNCTIONAL SULFHYDRYL REAGENT BISMALEIMIDOMETHYL ETHER*

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SUMMARY

Cysteine 80 and cysteine 117 of the \( \alpha \) subunit of the \textit{Escherichia coli} tryptophan synthetase have been cross-linked by use of the bifunctional reagent \( N,N'-\text{bis(maleimido-}

methyl) \) ether (BME). The sites of reaction were identified from the amino acid compositions of radioactive peptides purified from tryptic and tryptic-chymotryptic digests of \( \alpha \) subunit which had reacted with \( ^3\text{H}-\text{BME} \) to the extent of 1 mole of cross-linking reagent per mole of enzyme.

Hydrodynamic studies showed that no significant dimer formation had occurred. The electrophoretic and immunological properties of the enzyme are identical with those of untreated control preparations. Although the intramolecularly cross-linked monomers are apparently homogeneous by these criteria, BME-treated \( \alpha \) subunits are functionally heterogeneous in that 70\% of the independent indoleglycerol phosphate activity of the \( \alpha \) subunit has been lost after reaction of 1 mole of BME with 1 mole of \( \alpha \) subunit. In addition, 70\% of the protein in such preparations cannot form a functional \( \alpha_2\beta_2 \)-tryptophan synthetase complex with the \( \beta_2 \) subunit, as measured indirectly by enzymatic activity studies and directly in studies on \( \alpha_2\beta_2 \) complex formation in sucrose density gradient centrifugation experiments.

Two possible explanations for the observed functional heterogeneity of BME cysteine 80 to cysteine 117 cross-linked \( \alpha \) subunit preparations are advanced. These mechanisms may be interdependent. Two new asymmetric centers are introduced by the addition of the protein cysteiny1 thiols across the two maleimide double bonds of the bifunctional reagent.

The tryptophan synthetase of \textit{Escherichia coli} is composed of two types of separable protein subunits, originally designated the A and B proteins (2). The A protein, now referred to as the \( \alpha \) subunit, is monomeric with a molecular weight of 29,500 (3, 4). The B protein (molecular weight 99,000) (5, 6) has two identical, strongly associated polypeptide chains (5) and has been redesignated the \( \beta_2 \) subunit (6). The structure of the fully constituted tryptophan synthetase complex is that of an \( \alpha_2\beta_2 \) tetramer (3, 6).

The \( \alpha \) subunit catalyzes the reversible conversion of indole 3-glycerol phosphate to indole and D-glyceraldehyde 3-phosphate (7, 8) (indole 3-glycerol phosphate aldolase, EC 4.1.2.8). The \( \beta_2 \) component contains pyridoxal phosphate and catalyzes tryptophan synthesis from indole and L-serine (L-serine hydrolyase (adding indole), EC 4.2.1.20), and also catalyzes the deamination of serine and a number of other reactions (9, 10) in the absence of the \( \alpha \) subunit. The \( \alpha_2\beta_2 \) complex catalyzes both the
in GTP aldolase and l-serine hydroxylase reactions at higher rates than the isolated subunits, and in addition, InGP and serine are converted to tryptophan at a high rate without the detectable appearance of free indole as an intermediate (11, 12).

Previous studies on the effects of sulfhydryl reagents on the α subunit (13-16) have shown that the 3 cysteinyl residues at positions 80, 117, and 153 of the 267 amino acid sequence (17-23) are important to the tertiary structure and catalytic function of the enzyme.

Hardman and Yanofsky studied the effects of the sulfhydryl reagents, iodoacetate, and NEM, on the activity of the α subunit in the InGP aldolase reaction (13). Both reagents inactivated the enzyme completely, yet no inactivation occurred when InGP was present at substrate levels. A maximum of 3 moles of iodoacetate reacted per mole of α subunit, reacting equally with all 3 cysteinyl thiol groups. However, a maximum of only 1 mole of NEM reacted per mole of subunit, although radioactivity from SH-NEM was distributed equally between the 3 cysteinyl residues.

The observed substrate protection and failure of a 2nd molecule of NEM to react (although the initial NEM molecule might react with any of the three cysteines) and the fact that the less bulky iodoacetate molecules reacted readily and completely with all 3 residues, led Hardman and Yanofsky (13) to suggest that the α subunit might exist in a conformation in which the sulfhydryl groups were quite close to each other spatially. The determination of the complete sequence of the α subunit showed that the 3 cysteinyl residues were quite distant from each other in the primary sequence of the protein molecule, at positions 80, 117, and 153 (17-23). Thus the unique reactivity of these three sulfhydryl groups must reflect certain features of the three-dimensional structure of the α subunit in this portion of the protein molecule.

If the cysteine side chains were extremely close to each other in the three-dimensional geometry of the active site region, one might expect intramolecular oxidation to occur. However, disulfide bonds are found neither before, nor after treatment of the α subunit with NEM (13, 15).

To further examine the conformation of the α subunit in this critical region, the bifunctional reagent, BME, was chosen. This reagent may be considered as a bifunctional analogue of NEM, and has been used in conformational studies on hemoglobin (24, 25). In its fully extended configuration, BME has the potential to cross-link thiols 10 to 13 A between sulfur atoms. Hence, sulfhydryl groups too far apart from each other to be readily oxidized to disulfides, yet as close to each other as suggested by the data of Hardman and Yanofsky (13), might be covalently cross-linked by this reagent. This paper shows that 2 of the 3 cysteinyl residues can be cross-linked by BME under extremely gentle conditions.

### Materials and Methods

**Enzyme Preparations**—Wild type *E. coli* tryptophan synthetase α subunit was prepared as described by Malkinson and Hardman (15) but with elimination of the Sephadex step. Such preparations are homogeneous by the electrophoretic and hydrodynamic criteria applied by Malkinson and Hardman (15). Sonic extracts of the *E. coli* strain Δ2/F'/Δ2, which produce a defective α subunit, were the source of β subunit.

**Sulfhydryl Reagents**—BME was synthesized by the original procedure of Tawney et al. (26), from maleimide (Eastman) and reagent grade formaldehyde (J. T. Baker and Company). H-formaldehyde (New England Nuclear) was utilized for synthesis of tritiated BME. All preparations had the melting point reported by Tawney et al. (26) and showed the expected alkaline and neutral spectra. Quantitative elemental analysis of a representative sample of nonradioactive BME gave the following results.

\[
\text{C}_{14}\text{H}_{18}\text{N}_{2}\text{O}_{8}
\]

| Found: | C 50.0, H 3.4, N 11.9, O 33.9 |
|--------|-----------------------------|
| Calculated: | C 50.9, H 3.4, N 11.9, O 33.9 |

NEM was obtained from Nutritional Biochemicals, and NEM-1-14C from Schwarz BioResearch. DTNB was obtained from Aldrich.

**Other Chemicals**—Mercaptoethanol, pyridoxal phosphate, and DL-cysteine-HCl were obtained from Calbiochem. DL-Cysteine-1-14C was purchased from Amersham-Searle, Des Plaines, Illinois, and indole-2-14C from the Yolk Biochemical Company, Irvine, California. InGP was synthesized by the method of Creighton and Yanofsky (27), and InGP-14C was prepared as described from Hardman and Yanofsky (13).

**Protein Determination**—Protein concentrations were measured either by the method of Lowry et al. (28) or by a microbiuret method (29), with bovine serum albumin (Pentex, Inc., Kankakee, Illinois) as a standard. Values obtained by both methods agree with those obtained by amino acid analysis of the α subunit (15).

**Radioactivity Determinations**—14C and 3H activities were determined using a Nuclear Chicago model 3000 ambient temperature liquid scintillation counter operated at the balance point for each isotope. Aqueous samples were counted with Bray's solution (30) from which 1,4-bis(2-5-phenyloxazolyl)benzene (POPOP) and ethylene glycol were omitted (31).

**Enzyme Assays**—Activity of the α subunit in stimulating the conversion of indole and serine to tryptophan by the β subunit was assayed as described by Smith and Yanofsky (32). The conversion of InGP to indole and triose phosphate by the α subunit was assayed in a reaction mixture containing 20 mM potassium phosphate buffer (pH 7.0), and 0.5 mM InGP. When the InGP breakdown reaction was to be measured in the presence of excess β subunit, the reaction mixture was made 30 mM in hydroxylamine and 180 mM in NaCl (3). Indole formation was monitored either by the appearance of toluidine-extractable radioactivity when 14C-InGP was used as a substrate, or by colorimetric assay of indole as described by Smith and Yanofsky (32).

**Treatment of α Subunit with BME, NEM, and DTNB**—Before treatment with these reagents, α subunit preparations were dialyzed exhaustively against 20 mM potassium phosphate buffer (pH 7.0). The α subunit, 0.5 mg per ml, was treated with 0.5 mM NEM or BME in 20 mM potassium phosphate buffer (pH 7.0) at 21-22°C for 1 to 12 hours. The reaction was terminated by addition of mercaptoethanol in 20-fold molar excess with respect to BME or NEM, and the sample exhaustively dialyzed against 20 mM potassium phosphate buffer (pH 7.0). After protein and radioactivity measurements, the moles of reagent bound per mole of α subunit were calculated.
The DTNB titration method originally described by Ellman (33) was used under conditions of higher reagent concentration and neutral pH similar to those recommended by Janatova, Fuller, and Hunter (34). Titrations were performed on 10 to 30 moles of α subunit in 10 to 20 mM potassium phosphate buffer (pH 7.0). The reaction was initiated by the addition of 0.1 ml of a 10 mM DTNB solution made up in 0.1 M potassium phosphate buffer (pH 7.0). When DTNB titrations were performed in 4 to 5 M urea, the urea was added last. The final volume was 1.0 ml, and the optical density increase at 412 nm was used in the calculation of the free thiol content of the protein as described by Ellman (33).

Enzymatic Digestions—TPCK-trypsin and chymotrypsin (Worthington) were used to make tryptic and tryptic-chymotryptic digests of the α subunit as described by Helinski and Yanofsky (35). In tryptic-chymotryptic digestions, the protein was digested with TPCK-trypsin alone for 90 min, and then for an additional 90 min with both enzymes. Digests were freed of the 2 M urea-0.1 M ammonium carbonate digestion buffer by gel filtration on columns of Sephadex LH 20 or G 25, and lyophilized.

Peptide Purification—Tryptic digests of NEM- or BME-treated α subunit were chromatographed on columns, 133 × 0.9 cm, of Dowex 50-WX2, 200 to 400 mesh, at 37°. The peptides were eluted from the column by a convex gradient from 0.2 M pyridine, 5.0 M acetic acid, pH 3.1 (333 ml) to 2.0 M pyridine, 2.5 M acetic acid, pH 5.0 (666 ml), as described by Schroeder (36). Tryptic-chymotryptic digestions were chromatographed on columns, 133 × 0.9 cm, of Dowex AG 1-X2 at 37°, with the procedures for resin preparation described by Birshstein, Hussain, and Cobra (37). Peptides were eluted from the resin either with the complex pyridine acetate gradient described by Birshstein et al. (37) or by stepwise elution with 180 ml of 3% pyridine, followed by 180 ml of 0.1 M pyridine, 0.05 M acetic acid (pH 5.5), and, finally, a linear gradient between 180 ml of the latter buffer and 180 ml of 2.0 M pyridine, 2.0 M acetic acid (pH 5.0). Radioactive peptide peaks were concentrated and pyridine acetate removed by flash evaporation. Final purification of radioactive peptides was achieved by high voltage paper electrophoresis at pH 3.6 as described by Helinski and Yanofsky (35). Peptides were eluted from the paper with 10% acetic acid.

Peptide Amino Acid Compositions—All samples analyzed were homogeneous as judged by analytical scale paper electrophoresis. Samples were hydrolyzed for 22 to 24 hours in a vacuum at 110° in 5.7 N HCl, and amino acid analyses performed with the Beckman model 120 B amino acid analyzers of the Department of Biology service facility.

Peptide amino acid composition were calculated as follows. The amount of each amino acid found was first divided by the calculated input of radioactive 3H-BME or 14C-NEM peptide hydrolysate. The actual amount of peptide hydrolysate analyzed on the column was then calculated by averaging the recoveries of those stable amino acids which clearly represented 1-, 2-, or 3-fold multiples of a value close to, and usually 10 to 30% less than, the calculated amount of sample loaded onto the column, thus correcting for losses during transfer operations. As only limited amounts of material were available for analysis of most of the purified cross-linked peptides isolated, only 22- to 24-hour hydrolysates were performed, and the serine and threonine values were not corrected for losses caused by oxidation. Cysteine that had reacted with substituted maleimide reagents was determined as cysteiny1 succinic acid (S-cysteinosuccinic acid). It has been observed that the yield of this derivative after 22- to 24-hour hydrolyses is approximately 50% (38, 39).

RESULTS

Properties of BME-treated α Subunit

The choice of conditions for reaction of the α subunit with BME was based on several well recognized facts. Neutral pH and low reagent concentrations favor the specific reaction of substituted maleimide reagents with the cysteinyl sulfhydryl group of proteins, whereas reaction with the side chains of lysyl and histidyl residues, and with Nz-terminal amino groups, occurs under conditions of higher reagent concentration and more alkaline pH (39-41). Furthermore, low concentrations of protein and of bifunctional reagent should favor bifunctional reactions leading to the formation of intramolecular, rather than intermolecular cross-links (42).

When α subunit is treated with BME as described under "Materials and Methods" for 6 hours, a maximum of 1 molecule of BME reacts per mole of α subunit as a function of time; i.e., in the inset, represents the fraction of residual unreacted enzyme, E/Eo, as a function of time. E/Eo is plotted on a logarithmic scale, and has been calculated assuming the stoichiometry of the BME reaction with α subunit is 1:1.

FIG. 1. Time course of the reaction of BME with the α subunit. The α subunit was treated with 3H-BME as described under "Materials and Methods" for periods of 20 min to 6 hours. O, the number of moles of 3H-BME reacted per mole of α subunit as a function of time; i.e., in the inset, represents the fraction of residual unreacted enzyme, E/Eo, as a function of time. E/Eo is plotted on a logarithmic scale, and has been calculated assuming the stoichiometry of the BME reaction with α subunit is 1:1.

A 1:1 ratio of moles of bifunctional reagent bound per mole of protein is compatible with the formation of a single intra-
FIG. 2. Sedimentation velocity studies of BME-treated α subunit preparations. A and B are tracings made from contact prints of analytical ultracentrifuge plates (Schlieren optics). The numerals represent intervals in minutes at which the plate was exposed, starting at 1 min after attaining speed. Speeds: A, 59,780 rpm; B, 56,100 rpm. Protein concentrations (in 20 mM phosphate buffer, pH 7.0): A, 3.5 mg per ml; B, 3.6 mg per ml. s~θ~w~ values observed: A, 2.6; B, 2.7. C is a representative zone sedimentation profile of αH-BME-treated α subunit. A protein solution, 0.5 ml, containing 0.25 mg per ml of αH-BME-treated α subunit was layered on top of a 5 to 20% (w/v) 4.5-ml sucrose gradient in 0.1 M potassium phosphate buffer, pH 7.0, and centrifuged in a Spinco SW-50-L rotor in a model L-2 preparative ultracentrifuge at 39,000 rpm for 12 hours. Nineteen fractions (20 drops each) were collected and analyzed for αH content. O, αH counts per min per fraction, uncorrected for background of approximately 30 cpm.

TABLE I

| Preparation               | Sulphydryl content by DTNB titration |
|---------------------------|--------------------------------------|
|                           | No urea | 4.0 M urea |
| Untreated α subunit........ | 1.2 (± 0.3) | 2.0 (± 0.3) |
| BME-treated α subunit..... | 0.3 (± 0.2) | 1.3 (± 0.4) |

molecular cross-link in each protein molecule (42), yet formation of doubly cross-linked dimers, or simple monofunctional reaction with each protein molecule could also yield such ratios. A heterogeneous population of reacted and untreated α subunits might also fortuitously yield a net BME to α subunit ratio of 1.

Sedimentation velocity studies of BME-treated α subunit eliminated the possibility that dimers or higher order aggregates were present. Analytical ultracentrifugation with the Schlieren optics of the Spinco model E instrument, and analytical banding through 5 to 20% sucrose gradients showed that BME-treated α subunit sedimented with a single symmetric refractive index gradient boundary and as a single peak of recovered αH-BME radioactivity, respectively (Fig. 2). The calculated S values are identical with that of the native α subunit monomer (4).

BME-treated α subunit preparations were electrophoretically homogeneous and indistinguishable from the native α subunit with the polyacrylamide disc gel electrophoresis system described by Malkinson and Hardman (15), which they found capable of separating enzymatically active, singly labeled species of α subunit from inactive, multiply labeled species found after the reaction of the protein with NEM at pH 8.3.

To the limit of the resolving power of these hydrodynamic and electrophoretic criteria, the BME-treated α subunit preparations are free of intermolecularly cross-linked multimers, and are not detectably heterogeneous.

In the absence of dimer formation, the recovery of 2 moles of modified cysteine per mole of BME-treated α subunit would be strong evidence for an intramolecular cross-link between 2 cysteinyl residues. Total amino acid analyses of BME-treated α subunit after 24 and 48 hours of acid hydrolysis at 110°C yielded 1.1 moles of cysteinyl succinic acid per mole of α subunit. Since it has been reported that cysteinyl succinic acid is liberated in approximately 50% yield after 24 hours of acid hydrolysis of the reaction product of NEM and cysteine (38, 39), these data suggest that 2 cysteinyl thiols on each protein molecule had reacted with BME. The difficulties associated with accurately determining small amounts of this derivative in the presence of large amounts of other amino acids suggest the more conservative interpretation that at least 1, and perhaps as many as 2 of the α subunit cysteinyl residues have reacted with the reagent.

Similarly, titration of the remaining unreacted cysteinyl...
thiols of the BME-treated α subunit with the DTNB method (Table I) indicates that certainly one, and possibly two of the
three cysteinyl sulfhydryl groups have reacted with the bifunctional reagent.

In view of the possibility that BME might have reacted only monofunctionally with the α subunit, an experiment was de-
signed to test for the presence of unreacted maleimide rings bound covalently to the α subunit. This was done by deter-
mining the extent to which free 14C-cysteine could be bound covalently to the enzyme in the presence of urea. The specific
radioactivity of the 14C-cysteine added was sufficient to permit
detection of 0.1 mole of cysteine bound per mole of α subunit.

Results of such an experiment are shown in Fig. 3. During
the period from 40 min to 6 hours after the initiation of BME
treatment, no more than 0.1 to 0.2 mole of cysteine bound per
mole of α subunit could be detected. However, the interpreta-
tion of these data is complicated by the fact that α subunit
cysteinyl thiols, or a simple noncovalent binding of free
cysteine to the enzyme. In view of the exposure of the protein
to urea during the cysteine treatment and the exhaustive dialy-
sis afterwards, the first interpretation seems more likely.

The cysteine binding seen in the experiment described in
Fig. 3 may then represent residual oxidative or noncovalent
binding of cysteine, as well as the trapping of α subunits that
have reacted only monofunctionally with BME. The BME-
treated α subunit preparations must then contain at least 80 to
90% intramolecularly cross-linked monomer. It is also clear (II-A, II-B) were purified by preparative scale high voltage
paper electrophoresis. On the basis of the amino acid composi-
tions shown in Tables II and III, Peptides I-A and I-B were
assigned to the region of the protein sequence surrounding

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**Fig. 4.** Time course of the reaction of NEM with the α subunit.
The α subunit was treated with 14C-NEM as described under
"Materials and Methods" for periods from 20 min to 12 hours.

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### Table II

| Amino acid | Peptide I-A | Peptide I-B |
|------------|------------|------------|
| Lysine     | 0.7        | 0          |
| Histidine  | 0          | 0          |
| Arginine   | d          | 1          |
| Aspartic acid | 0.7  | 0          |
| Threonine  | 1.0        | 0.4        |
| Serine     | 0.5        | 0          |
| Glutamic acid | 2.9 | 2          |
| Proline    | 1.5        | 0          |
| Glycine    | 1.6        | 1.8        |
| Alanine    | 4.9        | 2.1        |
| Cysteine   | 0.4        | 1          |
| Valine     | 1.0        | 1.1        |
| Methionine | 0.6        | 0.2        |
| Isoleucine | 1.5        | 0.9        |
| Leucine    | 2.5        | 1.2        |
| Tyrosine   | 0          | 0.4        |
| Phenylalanine | 2.0 | 1          |

* Peptide including residues 70 to 90.
* Peptide including residues 72 to 90.
* Not analyzed.
* Lost.
* Cysteinyl succinic acid in approximately 50% yield (see "Materials and Methods").

### Localization of Sites of Reaction

**Sites of Reaction of Monofunctional Analogue, NEM—An α subunit preparation was treated with NEM for 12 hours under the same
conditions used for BME treatment. As shown in
Fig. 4, treatment with the monofunctional reagent under these
conditions proceeds at a rate similar to that of the BME α subunit reaction. However, if the time period is increased
beyond 6 hours, more than a net of one NEM may react per
mole of protein. Accordingly a preparation labeled to the extent
of 1.5 moles of NEM per mole of protein was chosen in hopes
of gaining the maximum amount of information about possible
sites of reaction by the bifunctional reagent from monofunctional
analogue studies.

This material was digested with TPCK-trypsin and the digest
desalted with Sephadex LH-20 with 70% recovery. The peak
fractions containing 80% of the radioactivity recovered from
the Sephadex LH-20 column were lyophilized and then chromato-
graphed on Dowex 50 with 64% recovery. Two sharp
well separated peaks, termed I and II, containing 49.5 and
50%, respectively, of the recovered radioactive NEM peptides,
were obtained.

High voltage paper electrophoresis at pH 3.6 showed that
each of these peaks was heterogeneous; each contained four well
separated ninhydrin-positive bands, of which two were in each
case radioactive. The radioactive peptides from the first Dowex peak pool (I-A, I-B) and the second Dowex peak pool
(II-A, II-B) were purified by preparative scale high voltage
paper electrophoresis. On the basis of the amino acid composi-
tions shown in Tables II and III, Peptides I-A and I-B were
assigned to the region of the protein sequence surrounding
Table III

| Amino acid | Peptide II-A | Peptide II-B |
|------------|-------------|-------------|
| Lysine     | 2.0         | 2.1         |
| Histidine  | 1.6         | 1.8         |
| Arginine   | 0.4         | 0.2         |
| Aspartic acid | 3.2       | 3.0         |
| Threonine  | 0.3         | 0.7         |
| Serine     | 0.2         | 1.1         |
| Glutamic acid | 3.2       | 4.2         |
| Proline    | 1.0         | 4.8         |
| Glycine    | 1.8         | 3.1         |
| Alanine    | 2.6         | 5.1         |
| Cysteine   | 0.5         | 0.2         |
| Valine     | 1.1         | 3.0         |
| Methionine | 0.3         | 0.2         |
| Isoleucine | 2.0         | 2.9         |
| Leucine    | 1.0         | 4.1         |
| Tyrosine   | 1.1         | 1.0         |
| Phenylalanine | 1.8       | 2.0         |

* Peptide including residues 89 to 119.
* Peptide including residues 91 to 144.
* Trace.
* Cysteinyl succinic acid in approximately 60% yield (see "Materials and Methods").

Table IV

| Amino acid | Peptide C* found | Peptide E-1, found | Peptide E-2, found | Theory |
|------------|------------------|-------------------|-------------------|--------|
| Lysine     | 1.7              | 2.3               | 1.9               | 2      |
| Histidine  | 0.0              | 0.3               | 1.3               | 0      |
| Arginine   | 1.1              | 2.1               | 2.1               | 2      |
| Aspartic acid | 4.0           | 7.2, 9.0         | 4.2, 3            |
| Threonine  | 1.1              | 0.7               | 0.9               | 1      |
| Serine     | 4.1              | 1.0               | 1.4               | 2      |
| Glutamic acid | 5.0           | 6.8               | 4.5               | 7      |
| Proline    | 3.9              | 4.2, 2.8          | 5.5               | 3      |
| Glycine    | 6.3              | 5.1, 7.2          | 3.1               | 3      |
| Alanine    | 9.5              | 7.3, 4.9          | 7.3               | 8      |
| Cysteine   | 1.0              | 1.1, 1.0          | 1.2               | 2      |
| Valine     | 8.0              | 6.2, 5.2          | 3.0               | 7      |
| Methionine | 0.1              | 0.3, 0.3          | 0.3               | 1      |
| Isoleucine | 3.4              | 3.1, 2.0          | 4.4               | 2      |
| Leucine    | 1.8              | 3.8, 3.0          | 3.8               | 4      |
| Tyrosine   | 0.4              | 1.7, 1.0          | 0.7               | 1      |
| Phenylalanine | 2.5           | 4.0, 3.2          | 2.8               | 4      |

* Only radioactive peptide found in Peak C.
* First radioactive peptide found in Peak E. Results of two long column analyses are presented.
* Second radioactive peptide found in Peak E. Results of two long column analyses are presented.
* Calculated composition of a cross-linked peptide including residues 70 to 99 and 109 to 139 of the α subunit.
* Lost.
* Cysteinyl succinic acid in approximately 50% yield (see "Materials and Methods").

Cysteine 80, II-A and II-B to the region surrounding cysteine 117. No radioactive peptides could be found by representing the reaction of NEM with cysteine 153. Attempts to recover additional radioactivity from the column by exhaustive washing were unsuccessful, and it is believed that the 20 to 30% losses during the purification steps represent nonspecific losses caused by the transfer and concentration steps involved.

**BME-Cross-linked Tryptic Peptides**—A TPCK-trypsin digest of 3H-BME-treated protein was desalted with Sephadex G-25 with 73% recovery. More than 50% of the radioactivity was eluted at the void volume of the column, suggesting that the cross-linked peptide fragments had molecular weights of at least 5000, although aggregation might also have occurred. This major radioactive fraction was lyophilized and chromatographed on Dowex 50 with 65% recovery. The radioactive peptides were eluted in the early part of the gradient, emerging in four peaks: Peak A, a sharp peak near the void volume of the column containing less than 10% of the recovered radioactivity; Peak B, a broad zone containing some 10% of the recovered radioactivity; and Peaks C, E, and F, closely associated peaks each containing approximately 25% of the recovered counts.

All radioactive fractions were then tested for heterogeneity with analytical scale high voltage paper electrophoresis, and, if needed, subjected to preparative scale purification by the same method. The amino acid composition of the Peak A material was compatible with monofunctional BME-labeling of lysine 36, resulting in the production of a large tryptic peptide containing residues 16 to 69 of the α subunit. Zone B and Peak F, even after purification attempts, gave amino acid analyses in which the ratios of most of the amino acids to the calculated input of BME peptide were extremely high, indicating that only very limited tryptic digestion had occurred. No specific peptide regions of the protein could be recognized on the basis of amino acid analyses of purified material from this part of the Dowex 50 chromatogram, and the quantities recovered were not sufficient to permit further digestion attempts with other proteolytic enzymes. The recovery of such large pieces of poorly digested reagent-treated protein was not observed with the NEM-treated α subunit tryptic digests. The large fragments may represent interference with the normal course of tryptic digestion of the α subunit resulting from the introduction of the intramolecular cross-link.

The peptides from Peaks C and E of the Dowex 50 chromatogram are large but nonetheless recognizable. A rigorous comparison of the composition of these peptides with all known tryptic peptide compositions indicate that these radioactive peptide results from a BME cross-linkage between cysteines 90 and 117 (Table IV). The results obtained after tryptic and chymotryptic digestion bear out this conclusion.

**BME-cross-linked Tryptic-Chymotryptic Peptides**—In three separate experiments BME-treated α subunit preparations were digested with trypsin and chymotrypsin in attempts to decrease the losses and size heterogeneity associated with the BME subunit tryptic peptides. Over-all yields on desalting by Sephadex G-25 chromatography were approximately 90% and nearly all radioactivity was eluted as a single peak at the void volume of the column. This material was pooled, lyophilized, and chromatographed on Dowex 1. Over-all recovery from the

**Materials and Methods**
TABLE V
Amino acid composition data for radioactive peptides purified by Dowex 1 column chromatography and high voltage paper electrophoresis from tryptic-chymotryptic digests of 3H-BME-treated a subunit

| Amino acid | Founda | Foundb | Foundc | Theoryd |
|------------|--------|--------|--------|---------|
| Lysine     | 7.6 (0.5) | 7.6 (0.5) | 7.6 (0.5) | 7.6 |
| Histidine  | 1.8 (0.9) | 1.8 (0.9) | 1.8 (0.9) | 1.8 |
| Arginine   | 0 (0)   | 0 (0)   | 0 (0)   | 0 |
| Aspartic acid | 1.9 (4.4) | 1.9 (4.4) | 1.9 (4.4) | 1.9 |
| Threonine  | 0.3 (0.4) | 0.3 (0.4) | 0.3 (0.4) | 0.3 |
| Serine     | 1.5 (2.0) | 1.5 (2.0) | 1.5 (2.0) | 1.5 |
| Glutamic acid | 4.5 (6.3) | 4.5 (6.3) | 4.5 (6.3) | 4.5 |
| Proline    | 5.8 (7.5) | 5.8 (7.5) | 5.8 (7.5) | 5.8 |
| Glycine    | 1.3 (1.1) | 1.3 (1.1) | 1.3 (1.1) | 1.3 |
| Alanine    | 6.5 (6.2) | 6.5 (6.2) | 6.5 (6.2) | 6.5 |
| Cysteineg  | 1.4 (1.2) | 1.4 (1.2) | 1.4 (1.2) | 1.4 |
| Valine     | 5.7 (8.0) | 5.7 (8.0) | 5.7 (8.0) | 5.7 |
| Methionine | 0 (0)   | 0 (0)   | 0 (0)   | 0 |
| Isoleucine | 0.6 (1.0) | 0.6 (1.0) | 0.6 (1.0) | 0.6 |
| Leucine    | 0.9 (0.9) | 0.9 (0.9) | 0.9 (0.9) | 0.9 |
| Tyrosine   | 2.1 (3.2) | 2.1 (3.2) | 2.1 (3.2) | 2.1 |

a Peptides purified by paper electrophoresis from oxidized and (in parentheses) oxidized portions of the material found in Peak II of the Dowex 1 chromatogram, Experiment 3.
b Peptides purified by paper electrophoresis from unoxidized and (in parentheses) oxidized portions of the material found in Peak III of the Dowex 1 chromatogram, Experiment 3.
c Unoxidized peptide sample purified by paper electrophoresis from pooled Peaks I to III of the Dowex 1 chromatogram, Experiment 1.
d Calculated composition of a cross-linked peptide containing residues 72 to 81 and 118 to 138 of the a subunit.
e Not determined.
f Data in parentheses represent amino acid analysis data for peptides oxidized with performic acid prior to high voltage paper electrophoresis.
g Cysteine determined as cysteiny1 succinic acid (in approximately 50% yield, see "Materials and Methods") for unoxidized samples, and as cysteic acid in the case of oxidized samples.

Dowex 1 columns was 90% or better, with 95% of the recovered radioactive peptide material emerging as three incompletely separated peaks in the early part of the gradient. Radioactive peptide material was purified further by high voltage paper electrophoresis. In some experiments, material from the Dowex 1 peak pools was subjected to performic acid oxidation by the method of Hir (43) before paper electrophoresis. No additional radioactive or ninhydrin-positive spots could be found when the electropherograms of performic acid-oxidized samples were compared with those of untreated samples, nor were the amino acid compositions or electrophoretic mobilities of the isolated radioactive peptides detectably altered. Performic acid oxidation of the cysteiny1 sulfur involved in a BME cross-link (by addition of the thiol group across the maleimide double bond) would be expected to produce a sulfone or sulfoxide, but not to result in cleavage of the thioether. Hence a BME cross-link would be stable to performic acid oxidation.

Tables IV to VI present amino acid analyses from unoxidized and oxidized peptide samples from these experiments. On the basis of these compositions, the cross-link represented by these peptides is between cysteines 80 and 117. No peptides were found which give amino acid analyses compatible with the involvement of cysteine 153 in a BME cross-link. Moreover, the BME tryptic-chymotryptic peptides all appear to be smaller fragments of the large tryptic BME ac subunit peptides presented in Table IV, which in turn approximate the sum of the cysteines 80 and 117 NEM-reacted peptides as presented in Tables II and III.

Catalytic and Immunochemical Properties of NEM- and BME-treated a Subunits

The relative specific activity of the a subunit treated with either BME or NEM falls to approximately 30% of the level found in untreated control preparations in all of the reactions tested, including not only reactions carried out by the unassisted a subunit but also reactions carried out by the a@ complex.

The time course of BME inactivation of the a subunit's enzymatic activity in the conversion of InGP to indole and triose phosphate in the presence of excess @ subunit is shown in Fig. 5. The time course for BME inactivation of the a subunit's ability to stimulate the @ subunit in the indole to tryptophan reaction is very similar. In both cases the rate of activity loss...
appears to be pseudo first order with respect to the concentration of active α subunit.

As the fractionally inactivated NEM- and BME-treated enzyme preparations were apparently homogeneous with respect to molecular weight and electrophoretic properties, the nature of the fractional remaining enzymatic activity was examined further with the method of Creighton and Yanofsky (3) for studying the association of the α and β subunits by sucrose density ultracentrifugation.

On sucrose density gradient centrifugation, in the presence of excess β subunits, both NEM- and BME-treated α subunits showed a distinct heterogeneity. Only 30 to 40% of the treated α subunits applied to the gradient were able to combine with the β subunit (Fig. 6). The remaining 60 to 70% sedimented in the same position as untreated α-subunit monomers in the absence of the β subunit.

The failure of a fraction of the treated material to form a functional tryptophan synthetase complex with the β subunit suggested that immunochemical techniques might be useful in the detection of structural heterogeneity in the BME- and NEM-treated α subunit preparations. If the tertiary structure of the inactive two-thirds of the treated α subunit population were grossly altered, it would be expected that immunochemical reactivity with antibody prepared against normal α subunit would also be lost.

If only the enzymatically active molecules in the treated population were immunochemically similar to the normal α subunit, then only 1 unit of antibody to normal α subunit would be required to neutralize 1 unit of enzymatic activity in the treated preparation. Fig. 7A shows that this is not the case, and that at least 2 antibody units are required to neutralize 1 enzyme unit in the treated material. This indicates the presence of a significant fraction of a subunits which retain immunochemically reactive tertiary structure, although they are catalytically inert. Precipitation of normal and treated α subunits in the presence of excess antibody was also studied. As shown in Fig. 7B, the quantitative precipitation reaction curves for all three types of α subunit, normal, NEM-treated, and BME-treated are superimposable. This is further evidence of a high degree of structural similarity between the normal and modified protein species.
The observed monofunctional reaction of BME with lysyl residue 35 in the 'H-BME-treated 01 subunit preparation used in the tryptic digestion experiments may be an anomaly, as reaction of this residue with either NEM or BME could not be detected in any of the other experiments.

The generation of functional heterogeneity in cysteine 80 to cysteine 117 cross-linked α subunit might occur by one of the following two mechanisms, or possibly by both acting in concert.

The addition of the thiol group of L-cysteine to the olefinic double bond of NEM has been shown to produce a new asymmetric center (39). Two diastereoisomeric forms of the product (S-1-ethyl-Z, 5 dioxopyrrolidin-3-yl) L-cysteine are produced. Extrapolation of these findings to the case of a symmetrical bifunctional maleimide reagent with free rotation between the maleimide rings leads to the conclusion that the addition of two protein cysteinyl thiols across the maleimide olefinic bonds must generate two new centers of asymmetry in the cross-link itself. Hence as many as four conformationally distinct cross-linked protein species might be formed, and the conformational heterogeneity thus introduced at or near the active site might be responsible for the functional heterogeneity observed.

A second mechanism which may possibly act together with the first involves the order of reaction of the two thiols with the bifunctional reagent. Although an experiment was performed with the purpose of trapping BME treated intermediates in which only one of the BME maleimide rings had reacted with an α subunit thiol, no intermediates in the cross-linking process could be detected. The reaction of the second maleimide ring must then follow quite rapidly after the initial reaction.
region, but also control which of the four possible cross-link conformations was finally attained by the BME bridge.

That such minor conformational changes in the sulfhydryl region of the α subunit should have such major consequences for the function of the β subunit both by itself and in the presence of the β subunit is not surprising, since the sulfhydryl groups of both α and β subunits are involved in maintaining the catalytic integrity of the unassisted subunits and in the interaction of the two types of subunits to form the serotonin synthetase complex (13-16). Moreover, it has been shown that the 01 of the pi subunit is not surprising, since the sulfhydryl groups of studies when these become available.

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