Structural and Functional Roles of the Cysteine Residues in the α Subunit of the *Escherichia coli* Tryptophan Synthetase. I. Structural Roles and Reactivity of the Cysteine Residues*

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ABSTRACT: Previously it was shown that different sulfhydryl reagents vary in the extent of their reaction with the tryptophan synthetase α subunit, which contains three cysteine residues. *N*-Ethylmaleimide, the larger reagent, reacts to the extent of 1 mole/mole of protein, while iodoacetate reacts completely with all three cysteine residues. The present work shows that other reagents similar in size to *N*-ethylmaleimide, such as *p*-hydroxymercuribenzoate and 5,5′-dithiobis(2-nitrobenzoic acid), react at the same rate and to the same extent as *N*-ethylmalemide in the absence of protein-denaturing agents. The absence of disulfide bonds either before or after *N*-ethylmaleimide treatment has eliminated this factor in the explanation of the reactivity of *N*-ethylmaleimide (a net binding of only 1 mole of *N*-ethylmaleimide and its nearly equal distribution on all three cysteine residues). The suggestion that several *N*-ethylmaleimide-labeled protein forms would result from *N*-ethylmaleimide treatment has been verified. In a fully *N*-ethylmaleimide-labeled preparation of the α subunit, there exist (a) unlabeled protein, (b) singly labeled protein (at cysteine-80, cysteine-117, and cysteine-153), and (c) doubly labeled protein (at cysteines-117 and 153). Indirect evidence indicates that the presence of unlabeled protein is due to its interaction and apparent masking by some of the labeled protein (cysteine-117, cysteine-153, or the doubly labeled derivative) during the labeling process.

Certain structural roles of the different cysteine residues are implied from immunochemical studies which indicate that *N*-ethylmaleimide labeling at either cysteine-117 or cysteine-153 causes an extensive loss of the native conformation of the protein. Labeling at cysteine-80, however, results in little or no detectable changes in the immunochemical properties of the protein.

The fully constituted tryptophan synthetase enzyme in *Escherichia coli* consists of two α and one β_2 subunit, $\alpha_2\beta_2$ (Creighton and Yanofsky, 1966; Goldberg *et al.*, 1966). This complex catalyzes reactions 1–3 (Crawford and Yanofsky, 1958; Crawford, 1960). The α and β_2 subunits alone can catalyze reactions 1 and 2, respectively, but at much lower rates than the $\alpha_2\beta_2$ complex.

$$\text{InGP}^1 \longrightarrow \text{In} \quad (1)$$

$$\text{In} \longrightarrow \text{Trp} \quad (2)$$

$$\text{InGP} \longrightarrow \text{Trp} \quad (3)$$

Chemical studies on structure and function relationships in the α subunit (Hardman and Yanofsky, 1965, 1967) had indicated a critical role of the cysteine-containing regions of the protein in its activity by itself (reaction 1), and had suggested an unusual arrangement of these sulfhydryl groups in the native enzyme. For example, *N*-ethylmaleimide, one of the reagents employed, reacted to the extent of 1 mole/mole of enzyme concomitant with complete enzymatic inactivation. The substrate, indoleglycerol phosphate, protected completely against alkylation and inactivation. However, all three cysteine had reacted apparently to an equal extent, suggesting that multiple forms of the enzyme were generated, each containing a different alkylated cysteine residue and each being enzymatically inactive. Moreover, it appeared that reaction of *N*-ethylmaleimide with any one of the cysteine residues prevented its reaction with the remaining cysteine residues in the same protein molecule.

Contrasting results were obtained with another reagent, iodoacetate. With this reagent, all three cysteine residues could be carboxymethylated. One explanation offered for this behavior was that the sulfhydryl groups were oriented relatively close to each other and once a molecule of *N*-ethylmaleimide had reacted with any one, it could hinder the reaction with the other two. In contrast, the smaller carboxymethyl group did not provide such steric interference and all three cysteine residues could be alkylated.

In this and the subsequent report (Malkinson and Hardman, 1969), a more rigorous examination of the reactivity of the cysteine residues was pursued, aimed at evaluating their roles in the structure of the protein and the effect of their modification on the ability of the protein to combine with the β_2 subunit and catalyze the activities of the fully constituted enzyme.

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[1] The reactions catalyzed by tryptophan synthetase are abbreviated as follows: InGP → Trp, the conversion of indoleglycerol phosphate and serine into Trp and glyceraldehyde 3-phosphate; InGP → In, the reversible conversion of indoleglycerol phosphate into indole and glyceraldehyde 3-phosphate; In → Trp, the conversion of indole and serine into Trp.

Methods and Materials

Purification of the α Subunit. Wild-type tryptophan synthetase α subunit was prepared from mutant 5927R38 (Somerville and Yanofsky, 1965) which is a partial revertant of an anthranilate synthetase mutant. When grown under conditions leading to derepression of the enzymes in the tryptophan pathway, this strain produces much higher levels of tryptophan synthetase than does the wild-type strain. For the preparation of the normal α subunit from large cultures of strain 5927R38, it was important to terminate growth at late-log phase. The specific activity of tryptophan synthetase was found to increase sharply at this stage of growth and to decrease abruptly at the onset of stationary phase to about one-half the maximum specific activity.

The purification procedure is essentially that described by Henning *et al.* (1962). In an attempt to remove a trace contaminant observed upon polyacrylamide disc electrophoresis of the preparations obtained by this procedure, several modifications were introduced. The most critical of these were the substitution of a Sephadex gel filtration step in place of the last DEAE-cellulose step and the addition of preparative polyacrylamide disc electrophoresis as the final step. These steps were performed as follows. *Sephadex step:* The active DEAE-cellulose fractions were combined into 150-mg batches. To each 100 ml, 45.9 g of ammonium sulfate was added; the suspension was stirred for 20 min and centrifuged at 13,000*g*. The precipitate was suspended in 0.02 M potassium phosphate buffer (pH 7.0) containing 0.005 M EDTA (pH 7.0) and 0.005 M β-mercaptoethanol, to a final volume of 4–6 ml and dialyzed 20 hr against 500 volumes of this same buffer. Sephadex G-75, Sephadex G-100, and Cellex-N-1 were mixed in a ratio of 1:3:1.25, and upon swelling, were poured to a column volume of 90 × 3 cm. The column was equilibrated with the above buffer and elution proceeded at a flow rate of 10 ml/hr; 5-ml fractions were collected. Fractions showing a positive spot test (Henning *et al.*, 1962) appeared after about 20 hr, and activity was distributed over seven to ten fractions. *Preparative polyacrylamide gel electrophoresis:* Active Sephadex fractions (50 mg) were pooled into volumes of 10–60 ml and electrophoresed on the Buchler preparative polyacrylamide gel electrophoresis apparatus. Conditions of electrophoresis (buffers, pH, etc.) were strictly identical with those described in detail in the Buchler "Poly-Prep" manual except that all buffers contained 0.5 mM dithiothreitol. The height of the resolving gel was 5 cm; the volume of the concentrating gel was 10–15 ml greater than the sample volume. No tracking dye was used. Upon introduction of the sample (adjusted to 5% sucrose), the current was adjusted to 45 mA, and after 15–20 min, was increased to 70 mA. Elution proceeded at a rate of 15–20 ml/hr and 20-min fractions were collected. Active spot test fractions appeared after about 7 hr and were distributed over eight to ten tubes. These were dialyzed 20 hr against 80 volumes of 0.05 M potassium phosphate buffer (pH 7.0) containing 0.5 mM dithiothreitol, with several changes. *Purity and yield:* Electrophoretic homogeneity was determined in an analytical polyacrylamide gel–buffer system similar to that used in the preparative procedure. Fractions exhibiting a single protein-staining band had specific activities of 4000–5000 units/mg and showed a single moving boundary during analytical ultracentrifugation. The tryptic fingerprint pattern and amino acid composition of the material were identical with those described (Guest *et al.*, 1967a) for the α subunit. Multiple band-containing fractions were pooled and concentrated with an Amicon Diaflo ultrafiltrator and recycled through preparative gel electrophoresis, resulting in additional electrophoretically homogeneous material. A yield of purified protein was generally 35–40% which compares favorably with the 25–40% yield in the Henning procedure.

Treatment with [^{14}C]N-Ethylmaleimide. Previously (Hardman and Yanofsky, 1965), labeling of the α subunit with [^{14}C]*N*-ethylmaleimide consisted of a 30-min incubation at 37° in 0.1 M ammonium carbonate buffer (pH 8.3). The α-subunit preparations purified as described above, however, were found to precipitate if labeling occurred at temperatures above 26°. In the experiments to be described here, the reaction was performed at 25° for 90 min. Before labeling, the enzyme was dialyzed against 100–400 volumes of 0.05 M potassium phosphate buffer (pH 7.0) for 10–15 hr to remove any reducing agent present. No loss of activity was observed after this dialysis. The conditions of labeling were otherwise identical with those of Hardman and Yanofsky (1965). The preparative polyacrylamide disc electrophoresis procedure for the fractionation of *N*-ethylmaleimide-treated protein is that used in the purification of the enzyme.

Treatment with 5,5′-Dithiobis(-2-nitrobenzoic Acid) and p-Hydroxymercuribenzoate. Protein was treated with 5,5′-dithiobis(-2-nitrobenzoic acid) according to the procedure of Ellman (1959). The reaction mixture contained 0.1 M potassium phosphate buffer (pH 7.0) or Tris-HCl buffer (pH 7.8), 0.2–0.35 mg of protein, and 6 M urea (when added) in a volume of 0.9 ml. The reaction was initiated with 0.1 ml of 0.01 M 5,5′-dithiobis-(-2-nitrobenzoic acid) and the absorbance at 412 mμ was monitored. Extinction coefficients were determined using L-cysteine and were found to be nearly identical with the published value of 13,600 (Ellman, 1959) with or without urea. *p*-Hydroxymercuribenzoate titrations were performed according to Henning *et al.* (1962).

In all experiments, protein was determined by the method of Lowry *et al.* (1951), using bovine serum albumin as a standard. This assay gave protein values identical with those obtained from amino acid analyses of the α subunit.

Isolation of [^{14}C]N-Ethylmaleimide-Labeled Cysteine Tryptic Peptides. The methods used for the isolation of these peptides have been described (Hardman and Yanofsky, 1965).

Analytical Ultracentrifugation. Sedimentation velocity experiments were carried out in a Beckman–Spinco Model E analytical ultracentrifuge, using synthetic boundary centerpiece and schlieren optics (Schachman, 1959). Protein samples were equilibrated with 0.1 M potassium phosphate buffer (pH 7.0) containing 0.5 mM dithiothreitol, by overnight dialysis. Protein concentration is noted in the legend to Figure 6. The rotor speed was 53,640 rpm, run at 20°. Sedimentation patterns were measured on a Mann Microcomparator.

Immunochemical Studies. Rabbits were immunized with α-subunit preparations which were pure electrophoretically and in the ultracentrifuge. Freund's adjuvant technique (Cohn, 1952) and multisite intrascapular injection were employed in three doses of 2 mg of protein; 1 week after the last injection the rabbits were bled and the serum was collected and stored at −18°. Nonimmune serum was obtained from each animal prior to immunization. In both the Ouchterlony immunodiffusion method (Crowle, 1961) and neutralization or cross-reacting material assays (Suskind, 1957), whole serum was used.

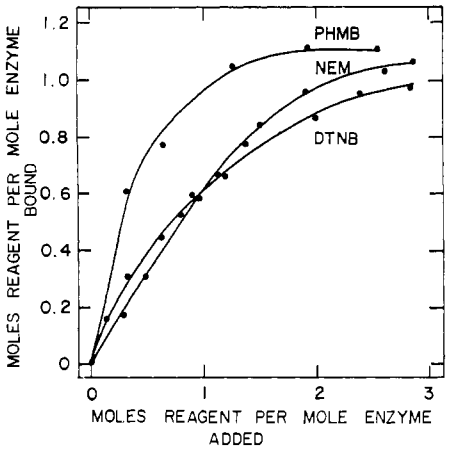

FIGURE 1: Binding of *N*-ethylmaleimide, *p*-hydroxymercuribenzoate, and 5,5′-dithiobis(-2-nitrobenzoic acid) to the α subunit. Each reagent was added to the protein at various ratios of reagent to protein and each reaction was assayed as described in Methods.

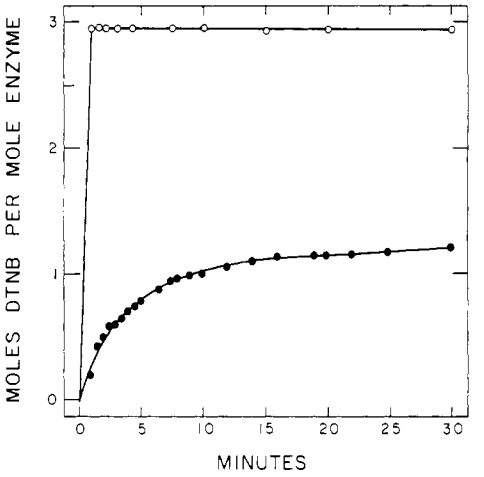

FIGURE 2: Kinetics of 5,5′-dithiobis(-2-nitrobenzoic acid) binding to the α subunit in the absence (●—●) and presence (○—○) of 6 M urea.

A unit of antibody is defined as that amount which neutralizes one unit of In → Trp activity.

Experimental Results

Reactivity of the Cysteine Residues with N-Ethylmaleimide, p-Hydroxymercuribenzoate, and 5,5′-Dithiobis(-2-nitrobenzoic Acid). As mentioned above, it was suggested that the reactivities of iodoacetate and *N*-ethylmaleimide with the α subunit differed as a result of their relative size. It was of interest, therefore, to explore reagents more comparable in size with *N*-ethylmaleimide, such as *p*-hydroxymercuribenzoate and 5,5′-dithiobis(-2-nitrobenzoic acid). Figure 1 indicates that all of these reagents react to the same extent. At nearly identical ratios of reagent to protein, only 1 mole is bound to the protein. Increasing the reagent/protein ratio to 5–10 does not result in substantially more binding under these conditions. The kinetics of the reaction with 5,5′-dithiobis(-2-nitrobenzoic acid) is shown in Figure 2. Similar rates have been observed also with *N*-ethylmaleimide and *p*-hydroxymercuribenzoate. With each reagent, there occurs a relatively rapid reaction (30–60 min at 25°) to a net of 1 mole of reagent/mole of protein. Longer incubations (2–4 hr) result in gradual increases to 1.3–1.4 moles of reagent bound per mole of protein.

The possibility that a disulfide bond in the untreated enzyme was causing this behavior does not seem likely since all three sulfhydryl groups are susceptible to carboxymethylation by iodoacetate under similar conditions (Hardman and Yanofsky, 1965). Furthermore, 5,5′-dithiobis(-2-nitrobenzoic acid) rapidly reacts with all three cysteine residues in 6 M urea (Figure 2). With *N*-ethylmaleimide, however, the extent of reaction is essentially unaltered in 6 M urea or 4 M guanidine hydrochloride. This difference between *N*-ethylmaleimide and 5,5′-dithiobis(-2-nitrobenzoic acid) reactivity in urea suggested that reaction with *N*-ethylmaleimide may induce the formation of disulfide bonds. Thus, 1 mole of reagent would be bound to one cysteine residue in each protein molecule, the remaining unalkylated residues being linked through a disulfide bond and not susceptible to reaction with *N*-ethylmaleimide. The results in Figure 3 indicate that this is probably not the case. In this experiment, samples of protein labeled with *N*-ethylmaleimide to varying extents in the usual way (lower curve) were titrated with 5,5′-dithiobis(-2-nitrobenzoic acid) in urea (middle curve). It is seen that neither at partial nor complete levels of *N*-ethylmaleimide labeling, do the number of 5,5′-dithiobis(-2-nitrobenzoic acid)-titratable sulfhydryl groups fall to zero. All the cysteine residues unsubstituted with *N*-ethylmaleimide are still available to react with 5,5′-dithiobis(-2-nitrobenzoic acid), and the total sulfhydryl groups (upper curve) titratable with both *N*-ethylmaleimide and 5,5′-dithiobis(-2-nitrobenzoic acid) is approximately three.

From these data, it would appear that in the absence of denaturing agents, these reagents all react similarly. The pattern

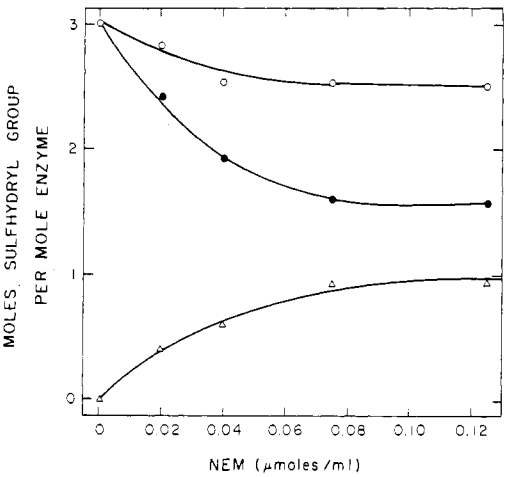

FIGURE 3: 5,5′-Dithiobis(-2-nitrobenzoic acid) binding to the α subunit in the presence of 6 M urea after previous treatment with *N*-ethylmaleimide. Moles of *N*-ethylmaleimide bound per mole of protein at different input *N*-ethylmaleimide concentrations (△—△). Moles of 5,5′-dithiobis(-2-nitrobenzoic acid) bound per mole of each of these *N*-ethylmaleimide-reacted samples determined in the presence of 6 M urea (●—●). Sum of the moles of sulfhydryl group reacted with *N*-ethylmaleimide and 5,5′-dithiobis(-2-nitrobenzoic acid) (○—○).

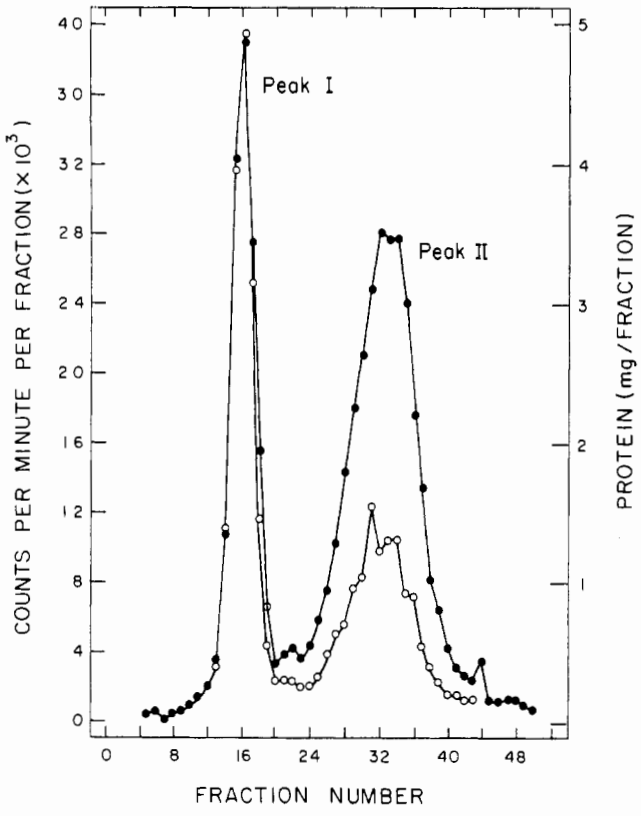

FIGURE 4: Preparative polyacrylamide gel disc electrophoresis of *N*-ethylmaleimide-labeled subunit; 30 mg of labeled protein (*N*-ethylmaleimide/protein = 1.06) was applied to the column. After dialysis, radioactivity (●—●) and protein (○—○) were determined.

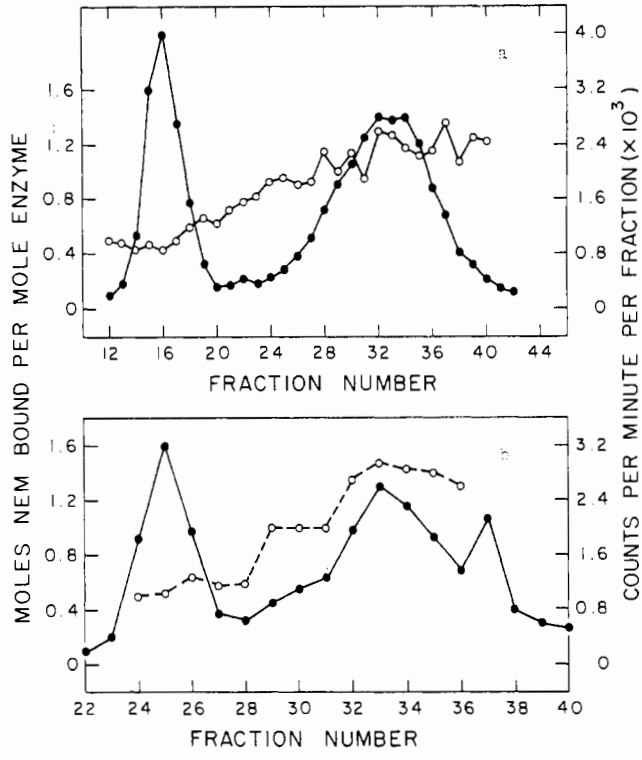

FIGURE 5: Variations in [^{14}C]*N*-ethylmaleimide/protein ratios of peak I and peak II; 20–30-mg samples of two preparations were fractionated by preparative polyacrylamide disc electrophoresis. After radioactivity (●—●) and protein determinations of the fractions, the *N*-ethylmaleimide/protein ratios were calculated (○—○). The initial *N*-ethylmaleimide/protein ratios of the two preparations were (a) 1.06 and (b) 1.05.

of enzymatic inactivation obtained with these reagents (Malkinson and Hardman, 1969) also supports this conclusion.

Polyacrylamide Disc Electrophoresis of N-Ethylmaleimide-Treated α Subunit. The fact that only 1 mole of *N*-ethylmaleimide could react with the protein and yet be distributed equally among the cysteine residues suggested that more than one *N*-ethylmaleimide-labeled derivative of the protein might be formed. Of the procedures sought to detect the presence of such forms, polyacrylamide disc electrophoresis methods appear to be the most satisfactory. Analytical gels of the α subunit fully labeled with either *N*-ethylmalemide, *p*-hydroxymercuribenzoate, or 5,5′-dithiobis(-2-nitrobenzoic acid) (*i.e.*, 1 mole/mole of protein) showed the presence of two protein bands. One of these is a relatively sharp band with the same mobility as the native enzyme; the other, a more diffuse, trailing band. It is not known whether this separation represents a difference in the net charge on the molecules resulting from *N*-ethylmaleimide binding at different cysteine residues or a difference in sieving effect on the gel as a result of either unfolding or aggregation of the molecules during electrophoresis. Regardless of the reasons, at least two types of molecules can be resolved by this procedure.

The preparative polyacrylamide disc electrophoresis pattern of *N*-ethylmaleimide-labeled α subunit (Figure 4) simulates that found on analytical gels in that two protein peaks were observed, one leading sharp peak, designated peak I, the other trailing and more diffuse, termed peak II. The protein elution patterns obtained with *p*-hydroxymercuribenzoate and 5,5′-dithiobis(-2-nitrobenzoic acid)-treated proteins were similar although less reproducible. All of the subsequent experiments utilizing this technique are concerned with *N*-ethylmaleimide-labeled protein. The characterization of some of the chemical and physical properties of each of these fractions is described below and the results are summarized in Table I.

It is seen that 30% of the ^{14}C is found in peak I, 70% in peak II; the protein is distributed equally between both peaks. As a consequence, the *N*-ethylmaleimide protein ratios differ markedly in the two peaks and vary substantially from unity. Figure 5 illustrates the variation in the *N*-ethylmaleimide/protein ratio for the individual fractions from two such runs. For peak I, this ratio was always between 0.4 and 0.6 whereas that in peak II ranged from 1.3 to 1.7. Pooled fractions of peaks I and II consistently contained average *N*-ethylmaleimide/protein ratios of approximately 0.5 and 1.5, respectively. In one such run, combined fractions of peak I and peak II were acid hydrolyzed. The amount of cysteinyl succinate (the derivative obtained by acid treatment of *N*-ethylmaleimide-reacted cysteine; Smyth *et al.*, 1964) was determined directly from the amino acid analysis; the amount of protein was calculated from the molar ratios of glycine, alanine, proline, valine, isoleucine, and leucine residues. On the basis of the amino acid analysis, the cysteinyl succinate/protein ratios for peak I and peak II were 0.57 and 1.31, respectively (Table I). These values agree well with the *N*-ethylmaleimide/protein ratios determined

TABLE I: Summary of Peak I and Peak II Characterization.

	Unfractionated *N*-Ethylmaleimide-Treated α Subunit	Peak I	Peak II
^{14}C distribution (%)	(100)	30	70
Protein distribution (%)	(100)	51	49
[^{14}C]*N*-Ethylmaleimide/protein	1.0–1.2	0.4–0.6	1.3–1.7
Cysteinyl succinate/protein		0.57	1.31
^{14}C distribution in Cys-tryptic peptides (%)			
TP-25	30	100	0
TP-29	37	0	53
TP-23	35	0	47
$s_{20,w}$ (S)	2.49	2.46	2.57
Immunochemical properties			
Cross-reacting material activity (%)		100	0
Precipitating activity		+	−

TABLE II: Cysteine Reactivity of Peak I and Peak II.

	Initial *N*-Ethylmaleimide/Protein (moles/mole)	Additional *N*-Ethylmaleimide Protein (moles/mole)	Additional 5,5′-Dithiobis(-2-nitrobenzoic Acid)/Protein (moles/mole)		Total (moles/mole)
			−Urea	+Urea	
Peak I	0.61	0.56			1.17
			0.30		0.91
				2.13	2.74
Peak II	1.45	0			1.45
			0		1.45
				1.18	2.63

by radioactivity and protein assays (0.58 for peak I; 1.45 for peak II).

Since the *N*-ethylmaleimide/protein ratio in peak I is always less than 1.0, both labeled and unlabeled protein are probably present. Tryptic peptide analysis of peak I material has shown that one of the cysteine-containing peptides, TP-25, contained all the radioactivity (Table I). The cysteine residue in this peptide corresponds to position 80 in the protein (Guest *et al.*, 1967b). Moreover, since the *N*-ethylmaleimide/protein ratio is approximately 0.5, this fraction must contain nearly equivalent amounts of unlabeled enzyme and enzyme singly labeled at cysteine-80.

The *N*-ethylmaleimide/protein ratio of peak II, on the other hand, implies that multiply labeled protein species are present. The only radioactive tryptic peptides found in these fractions were the cysteine-containing peptides, TP-23 and TP-29. The cysteine residues in these two peptides correspond to positions 117 and 153, respectively, in the protein. In addition, nearly equivalent amounts of each of these peptides were detected. Since the *N*-ethylmaleimide/protein ratio was about 1.5, peak II must contain two singly labeled protein forms (one labeled at cysteine-117, the other at cysteine-153) and a comparable amount of a labeled form (labeled at both cysteine-117 and cysteine-153).

An unexpected result was that, in an *N*-ethylmaleimide-saturated preparation of the α subunit, there exists unlabeled protein. The results shown in Table II indicate that some protein–protein interaction(s) occurring during the labeling process may account for this. Specifically, such interactions appear to involve unlabeled protein and the labeled protein found in peak II. Isolated peak I, which contains the unlabeled protein, will react further with both *N*-ethylmaleimide and 5,5′-dithiobis(-2-nitrobenzoic acid) in the absence of urea. The total amount of sulfhydryl group reacting with these two reagents is approximately one.

The nature of this interaction is unclear. Intermolecular disulfide formation in unfractionated *N*-ethylmaleimide-labeled protein appears to be ruled out from the previous 5,5′-dithiobis(-2-nitrobenzoic acid) titration data (Figure 3). Furthermore, 5,5′-dithiobis(-2-nitrobenzoic acid) titrations of isolated peak I and peak II in urea (Table II) exclude this type of intermolecular interaction between the different protein molecules within each peak. Peak I and peak II with *N*-ethylmaleimide/protein ratios of 0.61 and 1.45, respectively, bound 2.13 and

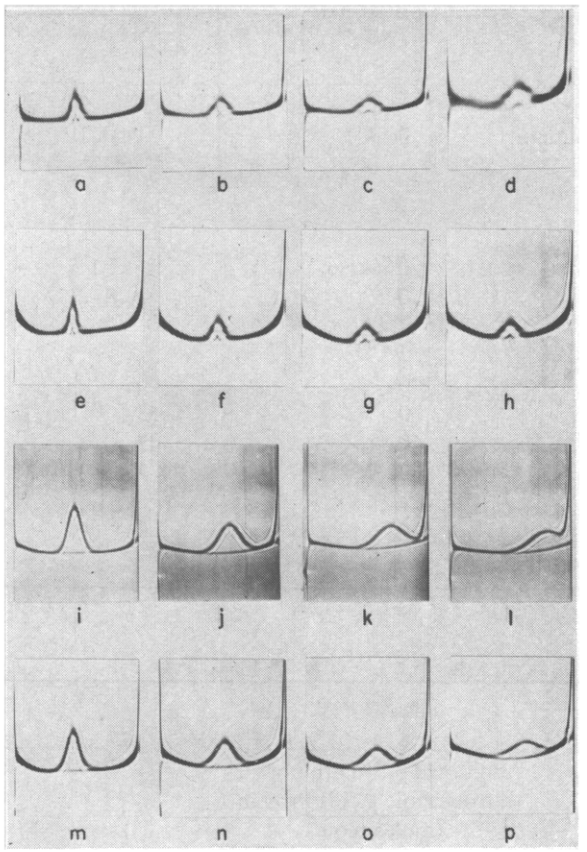

FIGURE 6: Sedimentation velocity centrifugation of untreated α subunit and *N*-ethylmaleimide-treated derivatives of the α subunit. The buffer used in all experiments was potassium phosphate buffer (pH 7.0). a–d, untreated α subunit (0.85 mg/ml) at 2, 10, 20, and 32 min. e–h, unfractionated *N*-ethylmaleimide-treated α-subunit (0.85 mg/ml) at 4, 12, 18, and 28 min. i–l, peak I (5.2 mg/ml) at 8, 32, 56, and 80 min. m–p, peak II (2.0 mg/ml) at 4, 16, 28, and 40 min.

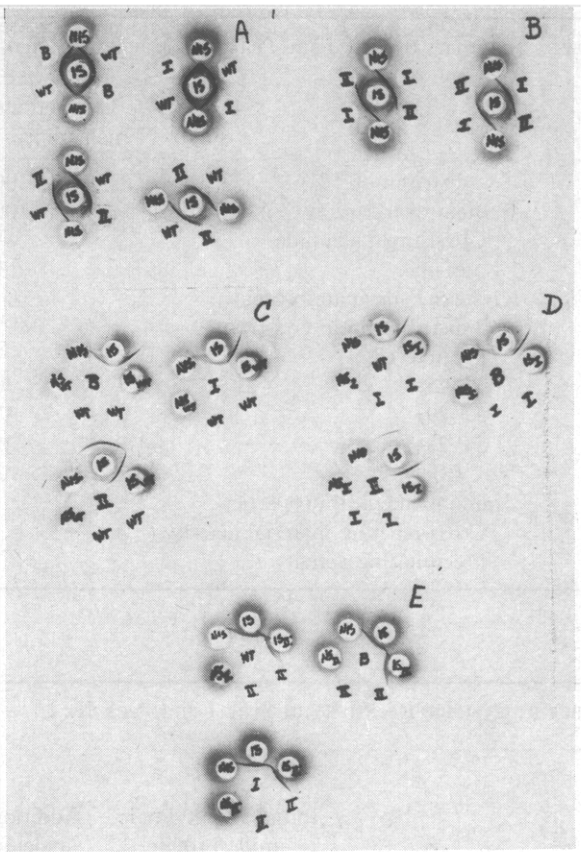

FIGURE 7: Ouchterlony immunodiffusion analysis of untreated α subunit and *N*-ethylmaleimide-treated derivatives of the α subunit. The following designations are used: IS, immune serum; NIS, nonimmune serum; WT, untreated α subunit; B, unfractionated *N*-ethylmaleimide-labeled α subunit; I and II, peaks I and II, respectively; IS_{WT}, IS_{I}, IS_{II}, immune serum mixed with untreated α subunit, peak I and peak II, respectively; NS_{WT}, NS_{I}, NS_{II}, nonimmune serum mixed with untreated α subunit, peak I, and peak II, respectively. Each well contained 0.02 ml of serum. Seven units of immune serum and 0.02 ml of nonimmune serum (diluted 1:1 with saline or the different enzyme preparations) were used throughout. With each enzyme preparation, 4- and 8-μg samples were used (the lower left quadrant of plate A and the left half of plate B contained 8 and 15 μg of peak II). In samples containing mixtures of serum and the enzyme preparations, 10–15 μg of each preparation was mixed at 0° with either type of serum and immediately put into the well.

1.18 additional moles of 5,5′-dithiobis(-2-nitrobenzoic acid). The total amount of reactive sulfhydryl groups is approximately three in each case. No other stable interaction was evident in preliminary sedimentation velocity experiments with any α-subunit preparation (Figure 6). $s_{20,w}$ values are similar for unfractionated *N*-ethylmaleimide-treated α subunit, peak I and peak II, and nearly identical with that of the native enzyme (Table I). These values agree well with that (2.7 S) previously reported for the native α-subunit monomer (Henning *et al.*, 1962).

Immunochemical Activity of N-Ethylmaleimide-Treated α-Subunit Preparations. Information regarding the structural roles of the cysteine residues has come from an examination of several immunochemical properties of *N*-ethylmaleimide-treated protein. In neutralization assays, peak I, which is enzymatically active (Malkinson and Hardman, 1969), and the native enzyme behaved identically. Peak II, which is enzymatically inactive (Malkinson and Hardman, 1969), was tested for its ability to block antibody-neutralizing activity. The results (Table III) indicate that at all levels of peak II tested, no loss in the neutralizing capacity of the antibody toward the normal enzyme was detected and suggest that peak II material contains no antigenic determinants corresponding to the active site of the native enzyme.

The ability of peak II to precipitate antibody or to block antibody precipitation by the normal protein was examined by the Ouchterlony agar diffusion technique (Figure 7). In plates A and B, a homologous reaction can be seen only between native protein, unfractionated *N*-ethylmaleimide-treated protein, and peak I. No precipitation was observed with peak II. In plates C, D, and E, serum was absorbed with the native protein, peak I, and peak II, respectively. It is seen, in plates C and D, that absorption with either the native protein or peak I totally eliminates further precipitation. The precipitin band observed between the absorbed antisera and the unabsorbed immune serum probably represents reaction of the excess of each enzyme preparation present in the absorbed serum. The results in plate E indicate that serum absorbed with peak II does not eliminate precipitation with the native protein, unfractionated *N*-ethylmaleimide-treated protein, or peak I.

TABLE III: Neutralization Assay of Peak II.

Peak II Added		Units Anti α Subunit Added	Units α Subunit Added[b]	Units α Subunit Recovered[b]	
ml	Theor CRM Units[a]			Found	Theor[c]
0	0	0	4.7	4.65	4.7
0	0	2.1	4.7	2.6	2.6
0.05	0.4	2.1	4.7	2.8	3.0
0.07	0.6	2.1	4.7	2.7	3.2
0.11	0.9	2.1	4.7	2.8	3.5
0.15	1.2	2.1	4.7	2.6	3.8
0.17	1.6	2.1	4.7	2.7	4.2
0.30	2.4	2.1	4.7	2.6	4.7

[a] Theoretical CRM (cross-reacting material) units which would be present if peak II was 100% CRM. [b] Units of activity with β_2 subunit in the In → Trp reaction. [c] Theoretical units of $\alpha_2\beta_2$ activity recoverable if peak II was 100% CRM.

Discussion

The results presented here have verified several predictions regarding the reactivity of the three cysteine residues in the α subunit and their roles in maintaining the normal structure of this protein. One of these predictions is concerned with the relative orientation of the sulfhydryl groups. Further evidence is presented to support the conclusion that different reagents will react differently depending, at least partly, on their relative size. Reagents similar in size to *N*-ethylmaleimide, such as *p*-hydroxymercuribenzoate and 5,5′-dithiobis(-2-nitrobenzoic acid), react to the same extent and at similar rates. One major difference between the reactivity of *N*-ethylmaleimide and 5,5′-dithiobis(-2-nitrobenzoic acid) occurs in urea. Undoubtedly, there is some loss in tertiary structure in 6 M urea as indicated by the ready reaction of 5,5′-dithiobis(-2-nitrobenzoic acid) with all three cysteine residues and by the loss of substrate protection against *N*-ethylmaleimide reaction (Hardman and Yanofsky, 1965). However, some normal conformation must still be retained which is impervious to total labeling by *N*-ethylmaleimide but not by 5,5′-dithiobis(-2-nitrobenzoic acid). 5,5′-Dithiobis(-2-nitrobenzoic acid) itself may be contributing to the unfolding of the cysteine-containing regions. One notable difference between *N*-ethylmaleimide and 5,5′-dithiobis(-2-nitrobenzoic acid) or *p*-hydroxymercuribenzoate is its lack of charge; whether this is a factor in its reactivity remains to be clarified.

Another earlier conclusion regarding the *N*-ethylmaleimide reaction was that multiple forms of the enzyme would be formed. The chemical nature of these forms, however, is different from that expected in that both unlabeled and doubly labeled enzyme were found. Figure 8 presents a diagrammatic representation of the results of *N*-ethylmaleimide labeling and fractionation. It can be seen that this model can account for most of the data.

At this time, none of the properties of peak I and peak II can be directly attributable to artifacts occurring during the electrophoresis procedure. The good correlation of all the data regarding ^{14}C, protein, [^{14}C]tryptic peptides, and disulfide content in unfractionated *N*-ethylmaleimide-treated protein and peak I plus peak II tend to support this conclusion. Other experiments, not reported here, have indicated that a similar fractionation can be achieved simply by labeling the protein at temperatures above 25°. Under these conditions, a major portion of the protein precipitates. A comparison of the properties of the soluble and insoluble protein (^{14}C and protein content, *N*-ethylmaleimide/protein ratios and the electrophoretic mobilities) indicate that all of the insoluble material

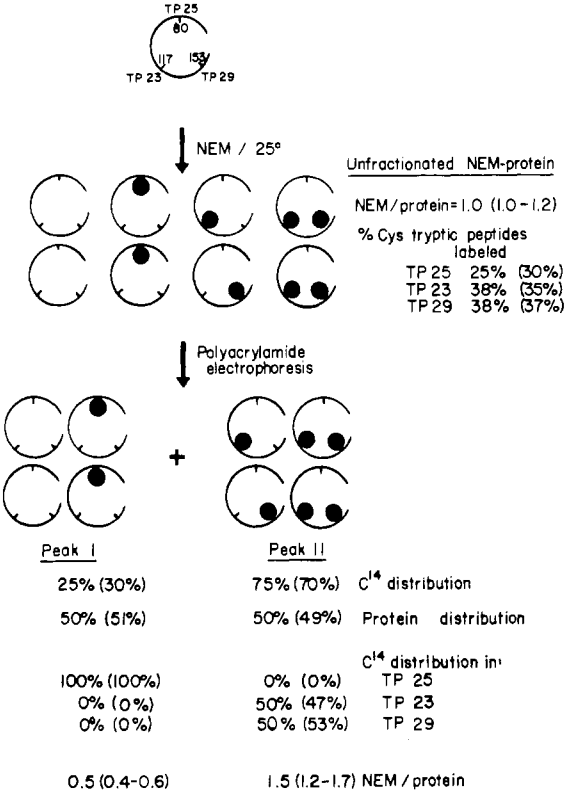

FIGURE 8: Diagrammatic representation of *N*-ethylmaleimide reaction with the α subunit at 25° and the subsequent fractionation on polyacrylamide disc electrophoresis. The partly closed circles refer to α-subunit protein molecules; the smaller solid circles refer to *N*-ethylmaleimide bound at the different cysteine residues. The results predicted from this model are presented together with the observed data (in parentheses).

is identical with peak II. The solubility properties of peak II (isolated in the usual way) at elevated temperatures is consistent with this behavior.

Certain structural roles can be tentatively assigned to the different cysteine residues in the protein. Labeling of cysteine-80 with *N*-ethylmaleimide apparently causes no major structural changes in the protein as judged by its immunochemical properties. In addition, it appears that once those protein molecules have been labeled at this position no additional changes occur such that other cysteine residues become labeled. In contrast, *N*-ethylmaleimide reaction at either cysteine-117 or cysteine-153 results in substantial loss of the normal conformation of the protein as indicated by a complete loss of immunochemical similarity to the native enzyme. The fact that doubly labeled protein forms contain *N*-ethylmaleimide at only these two cysteine residues suggests that these forms may have arisen from one or both of the singly labeled species found in peak II. The absence of immunochemical reactivity of all of the peak II material is consistent with the possibility that once *N*-ethylmaleimide has reacted at cysteine-117 or cysteine-153, these molecules unfold sufficiently to allow some additional reaction of *N*-ethylmaleimide at the other position. There is, however, no direct experimental evidence for this speculation. The ability of peak I and peak II to combine with the β_2 subunit (Malkinson and Hardman, 1969) is consistent with these conclusions regarding the conformation of these different *N*-ethylmaleimide-treated protein forms.

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