Studies on the Active Site of the A Protein Subunit of the Escherichia coli Tryptophan Synthetase*

JOHN K. HARDMAN† AND CHARLES YANOFSKY

From the Department of Biological Sciences, Stanford University, Stanford, California 94305

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The A protein subunit of the Escherichia coli tryptophan synthetase catalyzes the reversible conversion of indoleglycerol phosphate to indole and 3-phosphoglycerdehyde (1). This enzyme has been highly purified and consists of a single polypeptide chain with no associated metal (2, 3).

Extensive genetic and biochemical studies have been carried out in order to determine specific relationships between mutations in the A gene, the structural gene for the A protein, and the subsequent structure and function of the A protein. Thus, mutations in the A gene have been shown to result in the formation of altered A proteins which are enzymatically inactive; that is, they are unable to convert indoleglycerol phosphate to indole. In those defective proteins examined, a single amino acid difference from the wild-type enzyme could be demonstrated (4–9).

In addition, structural analyses of the A proteins from a number of revertant strains, which have a wild-type enzymatic behavior, also revealed single amino acid differences from the mutant enzyme (7, 9, 10). Such substitutions can occur at the site of the original mutant change or at a different site in the protein. These known amino acid changes which render a functional enzyme appear or a defective protein active lie along a 75-residue segment of the polypeptide chain (11). Other mutants have been obtained with altered sites that map at points throughout the A gene. In view of the observed colinearity between mutations in the A gene and alterations in one segment of the A protein (11), it would appear that amino acid substitutions throughout the A protein render it inactive.

Although primary structure studies may give some information on critical amino acid sequences required for maximum activity of the enzyme, they can be interpreted more readily in the light of detailed knowledge of those portions of the primary structure of the wild-type enzyme which are required for substrate binding and turnover. This paper describes initial studies aimed at detecting those amino acid residues at or near such site or sites on the protein. Hopefully, such information will eventually suggest explanations for the absence or presence of activity in the mutant and revertant A proteins, respectively. The results obtained indicate a requirement for one to two intact cysteine residues for maximum enzymatic activity and suggest the possible presence of a histidine and methionine residue at or near the site of substrate binding. In addition, some unusual properties of the sulfhydryl groups of the enzyme are described.

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† National Academy of Sciences Postdoctoral Research Fellow; Postdoctoral Fellow of the United States Public Health Service.

EXPERIMENTAL PROCEDURE

Enzyme Preparations—E. coli, strain B8, has been used as the source of wild-type or normal A protein. This strain forms very low levels of an altered B protein and high levels of normal A protein. The purification procedure is described elsewhere (2). In one of the experiments reported here, the enzyme preparations used consist of concentrated fractions obtained from the second DEAE-cellulose column step. Those fractions with a specific activity of 3500 units per mg or greater (generally greater than 85% A protein) were treated with 60 to 70% saturated ammonium sulfate. The precipitate was dissolved in 5 mM potassium phosphate buffer (pH 7.0) and dialyzed against this same buffer for 3 to 4 hours before use.

For the enzymatic preparation of InGP, a partially purified preparation from extracts of mutant B8 was used as the source of A protein (low specific activity fractions from the DEAE-cellulose column step (2)); the B protein was also slightly purified from extracts of mutant A2 which forms a high level of normal B protein and no detectable A protein (12). The B protein preparation consists of a 0 to 33% saturated ammonium sulfate fraction of a protamine sulfate-treated crude extract of this organism. Alternatively, extracts of mutant T-3 have been employed as the source of both A and B proteins. This mutant is blocked in the synthesis of anthranilate and forms high levels of normal A and B proteins (13). The limited purification procedure described above for the B protein was also carried out on crude extracts of this organism.

Activity Assays—Although the enzymatic activity of the A protein alone in the InGP to indole reaction is only a fraction of that obtained with the A protein-B protein complex, a sensitive and reproducible assay for the A component in this reaction was achieved under the following conditions. The assay mixture contained potassium phosphate buffer, pH 6.5, 50 μmoles; InGP-2-14C, 0.4 μmole (usually 30,000 c.p.m. per μmole); and enzyme in a volume of 1.0 ml. After incubation at 37°, the labeled indole formed was extracted with 3 ml of toluene and an aliquot of the toluene layer was counted in a Packard liquid scintillation counter. The activity was linear with time (up to 40 minutes) and with enzyme concentration over a wide range. Normally, assays were carried out with 0.05 to 0.2 mg per ml of purified A protein. Under these assay conditions, there was found a broad pH optimum for activity from pH 6.2 to 7.5 which falls off sharply at both extremes of this range. The K_m for InGP as determined by Lineweaver-Burk analysis was 0.47 mM.

The abbreviations used are: InGP, indoleglycerol phosphate; EM, N-ethylmaleimide; and HMB, p-hydroxymercuroribenzoate.
No metal requirement or detectable effect of sulfhydryl compounds (2-mercaptoethanol, glutathione, cysteine) has been observed.

**Materials**—EM-1-14C was purchased from Schwarz BioResearch, Inc.; iodoacetate-1-14C and indole-2-14C from New England Nuclear Corporation. Unlabeled iodoacetic acid was freshly prepared immediately before use. InGP was synthesized enzymatically from indole and glucose 1,6-diphosphate plus aldolase. The reaction mixture contained potassium phosphate buffer, pH 7.0, 0.1 M; indole, 0.5 mM; glucose 1,6-diphosphate (trisodium salt), 3 mM; crystalline aldolase, 0.1 mg per ml; and partially purified preparation or preparations of A and B proteins, 100 units of each per ml. The volume was usually 2 to 3 liters. Incubation was for 1 hour at 37° at which time InGP formation, as measured in the periodate assay (14), had ceased. The isolation procedure for this compound has been described previously (15). InGP-14C was prepared in the manner described above with indole-2-14C in place of indole. 3-Phosphoglyceraldehyde was obtained after hydrolysis of the monobarium salt of 3-phosphoglyceraldehyde-3-phosphate diethylacetal. The hydrolysis conditions were as follows. The barium salt (20 mg) was suspended in water (2.0 ml). Approximately 200 mg of dry Dowex 50-H+ (200 to 400 mesh) were added; the mixture swirled for a few minutes and then filtered. The resulting solution was incubated at 37° for 18 hours and neutralized with crystalline NaHCO3. The concentration of 3-phosphoglyceraldehyde was determined enzymatically with the triosephosphate dehydrogenase system.

**RESULTS**

*Effect of pH on InGP Interaction with Enzyme*—A method of identifying an amino acid residue involved in enzyme activity has been suggested which does not involve any covalent labeling of the protein with a chemical reagent (16, 17). Instead, advantage is taken of the different dissociation properties of the amino acid side chains in a protein. Since these pK values differ appreciably, a tentative identification of an amino acid residue involved in substrate binding or in attaining maximum reaction rate can be made by noting the variation of $V_{max}$ or $K_m$ with pH. Fig. 1 shows the results of such an experiment in which the $K_m$ for InGP (expressed as the pKm) was observed at several pH values. It can be seen that there is a sharp increase in the affinity for the substrate between pH 5 and 6. Assays for activity below pH 5 could not be performed since the enzyme precipitates from solution under the conditions of assay.

*Effect of EM*—Amino acid analyses of performic acid-oxidized A protein had indicated that there were three cysteic acid residues per mole of protein (2). In the unoxidized enzyme, only one of these existed as a readily titratable free cysteine residue (2). The role of the free sulfhydryl group in the activity of the protein was examined with several sulfhydryl group reagents. Preliminary experiments indicated that both HMB and EM were indeed potent inhibitors of activity. The enzyme was 50% inhibited at 0.02 mM HMB or 0.05 mM EM. Although the inhibition observed with HMB could be largely (about 70%) reversed by a number of sulfhydryl compounds such as thioglycolate or 2-mercaptoethanol, EM inhibition was not similarly affected. Since the latter inhibitor forms a relatively stable thioether with sulfhydryl groups, it was employed in an attempt to correlate the loss of activity with the loss of free cysteine residues. Fig. 2A shows the extent of activity loss as a function of EM concentration. InGP, present during EM treatment, prevents this loss to a large degree. The extent of labeling with EM-14C under identical conditions of EM concentration in the presence and absence of substrate is shown in Fig. 2B. Approximately 1 mole of EM per mole of enzyme was found under conditions of complete enzyme inactivation. The substrate prevents the labeling reaction also. Fig. 2C shows the loss of activity relative to the extent of labeling with EM in the absence of InGP. It can be seen that enzyme inactivation occurs concomitantly with the loss of one free cysteine residue.

Although InGP, the substrate in the forward reaction, has been used in the majority of experiments to show the specific effect of the substrate on protein modification procedures used, the products of the reaction, indole and 3-phosphoglyceraldehyde, have also been found to exert a protective effect. This was found with both EM and iodoacetate treatment of the enzyme. An important point in this protection phenomenon, at least with EM treatment, is that both products must be present to prevent labeling. This can be seen in Fig. 3. At concentrations equivalent to that of InGP, a mixture of indole and 3-phosphoglyceraldehyde will effect an equivalent protection of EM labeling, whereas either product alone produces no decrease in labeling. The possibility that the protection observed with these compounds reflects that of InGP which is formed during the course of incubation has not been ruled out, although this seems unlikely in view of the relatively low turnover number of the A protein alone in this reaction. It can be calculated that, at most, 0.1 to 0.2 μmole of InGP could be formed under these conditions. Other experiments have shown that this concentration of InGP gives no significant protection.

*Tryptic Digestion of EM-labeled Enzyme*—In order to determine which portion of the protein contains the EM-labeled cysteine residue, tryptic digestion of EM-labeled protein and isolation of the labeled peptide were undertaken. The three cysteic acid residues in performic acid-oxidized enzyme appear in three different tryptic peptides, TP-25, TP-29, and TP-23. When these peptides were isolated following labeling of the protein to the extent of 1.1 moles of EM-14C per mole of enzyme, the surprising finding was obtained which is shown in Table I. It is seen that the radioactivity is equally distributed among all three peptides. In addition, the ratio of EM-14C to cysteinylsuccinyl thioether, the derivative obtained after acid hydrolysis, is approximately 1.0 for each peptide.

![Fig. 1](http://www.jbc.org) The effect of pH on the pKm of InGP. The Km values were determined by Lineweaver-Burk analysis.
FIG. 2. A, the effect of EM on enzyme activity of the A protein in the presence and absence of InGP. EM treatment of the enzyme was carried out under the following conditions. The reaction mixture contained: ammonium carbonate buffer, pH 8.3, 100 μmoles; enzyme, 1.0 mg of protein; EM; and InGP, 7 μmoles, when added, in a total volume of 1.0 to 1.3 ml. After reaction for 30 minutes at 37°, the unreacted EM was removed by overnight dialysis against 5 mM potassium phosphate buffer (pH 7.0). Aliquots of each sample were assayed for enzyme activity. B, the labeling of the A protein by 14C-EM in the presence and absence of InGP (7 mM). Identical conditions for reaction with 14C-EM were employed. The unreacted 14C-EM was removed either by dialysis or by precipitation of the protein with triphloroactic acid (10% final concentration) in the presence of a 100-fold excess of the unlabeled reagent. In the latter case, the precipitate was washed with 10% triphloroactic acid and dissolved in 0.1 M ammonium carbonate buffer (pH 8.3). Aliquots of each sample were assayed for protein and radioactivity, and the moles of reagent taken up per mole of enzyme were calculated from these data. C, correlation of activity loss with labeling of the A protein by 14C-EM.

Two possible explanations for this observation were considered. The enzyme may exist in three forms prior to reaction with EM, each of which would have a different free cysteine residue, the other two residues being linked in a disulfide bond. Alternatively, there may be only one species of protein which contains three free cysteine residues, but reaction of any one with EM blocks reaction with the other two. To distinguish between these possibilities, an attempt was made to label all three cysteine residues with EM in the presence of urea or guanidine. In this experiment it was found that there was no increase in the moles of EM fixed per mole of enzyme in the presence of 9 M urea or 4 M guanidine, even at concentrations of EM 5- to 10-fold higher than those normally used to label one sulfhydryl group. This result might be considered to favor the first model although it would not rule out the other altogether.

Effect of Iodoacetate—Another sulfhydryl-binding reagent, iodoacetate, was employed to investigate further the unusual

FIG. 3. The protection of 14C-EM labeling of the A protein in the presence of no additions; indole, 8.5 mM; 3-phosphoglyceraldehyde, 7.5 mM; indole plus 3-phosphoglyceraldehyde; or InGP, 7.1 mM.

TABLE I

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Total radioactivity</th>
<th>14C-EM per mole of cysteinyllysylthioether</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP-25</td>
<td>30</td>
<td>0.95</td>
</tr>
<tr>
<td>TP-29</td>
<td>36</td>
<td>1.46</td>
</tr>
<tr>
<td>TP-23A†</td>
<td>33</td>
<td>1.10</td>
</tr>
<tr>
<td>TP-23B†</td>
<td>33</td>
<td>1.04</td>
</tr>
</tbody>
</table>

* The amino acid composition of each peptide is as follows:
TP-25: (Cys, Thr, Glu, Pro, Gly, Ala, Val, Met, Ileu, Leu, Phe) Arg.
TP-29: (Cys, Asp, Pro, Ala, Val, Ileu, Leu, Phe, His) Arg.
TP-23: (Cys, Asp, Glu, Gly, Ala, Ileu, Tyr, Phe) Lys.
† TP-23 appeared in two different fractions during Dowex 1 column chromatography. Since each gave an identical amino analysis after acid hydrolysis, it is presumed that the difference resides in the degree of amide hydrolysis during purification.

The methods used for tryptic digestion of the treated enzyme and for the isolation of the pertinent peptides after Sephadex gel filtration and Dowex 1 chromatography have been described in detail (8). Final purification of TP-25 was achieved by paper chromatography (18); TP-29 and TP-23 by high voltage paper electrophoresis (18). The peptides were eluted from paper with 0.05% 2-mercaptoethanol and hydrolyzed in 6 M HCl containing 2-mercaptoethanol to reduce excessive oxidation of the cysteine derivatives.
reactivities of the sulfhydryl groups in this protein and their roles in enzyme activity. Experiments similar to those described with EM except that a 90-minute incubation period was used. B, the labeling of the A protein by \(^{14}\)C-iodoacetate in the presence and absence of InGP (2 mM). See A for experimental detail.

Fig. 4. A, the effect of iodoacetate on enzyme activity of the A protein in the presence and absence of InGP (2 mM). The reaction conditions for treatment with iodoacetate were similar to those described with EM except that a 90-minute incubation period was used. B, the labeling of the A protein by \(^{14}\)C-iodoacetate in the presence and absence of InGP (2 mM). See A for experimental detail.

In view of the fact that several amino acid residues can be carboxymethylated by iodoacetate, it was necessary to determine which amino acid residues were reacting under the conditions used. The carboxymethyl derivatives of the various amino acid residues which can react are identifiable by their elution pattern on an amino acid analyzer. Thus, the enzyme was carboxymethylated to the extent of 2 moles of carboxymethyl derivative per mole of enzyme and the protein examined after acid and alkaline hydrolysis. It was found that the 2 moles of iodoacetate-\(^{14}\)C on the enzyme existed as two residues of carboxymethylcysteine. This observation is in contrast to that made previously with HMB and EM which indicated that a net of only one cysteine residue in the enzyme could react. This point was further examined in experiments with iodoacetate. The data in Fig. 4 show that, at a 4-fold higher concentration of iodoacetate, the protein could be carboxymethylated to a net of 3 moles of iodoacetate-\(^{14}\)C per mole of enzyme and that, again, InGP effects a net protection of 1 mole. That only cysteine residues are reacting with iodoacetate is indicated by the data in Table II. It thus appears that each cysteine residue is, in fact, approximately equally reactive toward iodoacetate and that the substrate will protect any one of the three.

If this is the case, it is difficult to reconcile the results of iodoacetate labeling with the enzyme inactivation data described in Fig. 4. The uptake of 1 and 2 moles of iodoacetate per mole of

**TABLE II**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Methionyl</th>
<th>Lysyl</th>
<th>Histidyl</th>
<th>Carboxymethyl-cysteine</th>
<th>i(^{14})C-Iodoacetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Alkaline hydrolysis</td>
<td>4.7</td>
<td>4.9</td>
<td>5.3</td>
<td>0</td>
<td>1.28</td>
</tr>
<tr>
<td>2. Acid hydrolysis</td>
<td>14.9</td>
<td>4.3</td>
<td>0.82</td>
<td>0</td>
<td>0.91</td>
</tr>
<tr>
<td>3. Acid hydrolysis</td>
<td>15.9</td>
<td>4.6</td>
<td>1.50</td>
<td>0</td>
<td>1.09</td>
</tr>
</tbody>
</table>

**Fig. 5** (left). The more extensive labeling of the A protein by \(^{14}\)C-iodoacetate in the presence and absence of InGP (6 mM).

**Fig. 6** (right). The kinetics of labeling of the A protein by \(^{14}\)C-iodoacetate (15 mM) and of activity loss.
enzyme results in 50 and 100% loss of activity, respectively. Since each cysteine residue appears to be equally reactive toward iodoacetate, do these values of iodoacetate uptake represent molecular species of the enzyme having only one or only two substituted cysteine residues? Reaction of the unsubstituted enzyme, which results in the uptake of 1 mole of iodoacetate, would be much more rapid than that of the monosubstituted enzyme with the second mole of iodoacetate. Alternatively, do the levels of 1 or 2 moles of iodoacetate per mole of enzyme represent the average obtained in a population containing, simultaneously, species of the enzyme which have 0, 1, 2, and 3 moles of carboxymethylcysteine? In this case, similar rates of reaction with iodoacetate might be expected with the un-, mono-, and disubstituted enzyme. An examination of the kinetics of the iodoacetate reaction has helped to clarify this point somewhat (Fig. 6). It is seen that there is a significant difference in rate of carboxymethylation after 1 mole of iodoacetate has reacted. This striking difference in rate is consistent with the interpretation that, at least at the level of formation of one carboxymethyl cysteine, there exist, predominately, species of the enzyme with only one cysteine substituted. Whether, at this level of carboxymethylation, each of the cysteine residues has reacted is not yet known, although it seems probable.

It is also seen in this experiment that the loss of activity precisely parallels carboxymethylation to the extent of 2 moles per mole of enzyme. These results, together with those represented in Fig. 4 and the apparent difference from the EM experiments, will be discussed below.

Effect of Photo-oxidation—To examine the possible roles of other amino acid residues in enzyme activity, another relatively nonspecific treatment of the protein was undertaken. Photo-oxidation, in the presence of methylene blue, has been commonly employed as a nonspecific method of protein alteration. This treatment, originally described by Weil et al. (19, 20) was shown to oxidize cysteine, histidine, methionine, tyrosine, and tryptophan residues. Accordingly, the effect of photo-oxidation on the enzymatic activity and the amino acid content of the A protein was examined. Relatively mild conditions of photo-oxidation have been found to result in the loss of enzymatic activity of the A protein. Figs. 7 and 8 present the results of these experiments. In Fig. 7A, it is seen that there is essentially complete loss of activity after 60 minutes of photo-oxidation. The presence of substrate, InGP, prevents this activity loss to a large degree. Control samples, incubated in the dark, showed no loss of activity.

The relative rates of loss of oxidizable amino acid residues in the protein were then measured in the same samples in which enzyme activity loss was estimated. Histidine and tyrosine were determined by amino acid analysis after acid hydrolysis; methionine after alkaline hydrolysis. Tryptophan was not considered since the A protein does not contain this amino acid. This

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Total radioactivity</th>
<th>$^{14}$C-Iodoacetate per mole of carboxymethylcysteine</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP-35</td>
<td>29</td>
<td>0.75</td>
</tr>
<tr>
<td>TP-29</td>
<td>33</td>
<td>0.73</td>
</tr>
<tr>
<td>TP-23</td>
<td>38</td>
<td>0.73</td>
</tr>
</tbody>
</table>

Fig. 7. A, the kinetics of activity loss as a result of photo-oxidation in the presence and absence of InGP. The procedure employed was as follows: to Pyrex tubes (1.3 X 10 mm) were added: Tris-HCl buffer, pH 7.0, 100 μmoles; methylene blue, 0.02 mg; and enzyme, 5.5 mg of protein, in a total volume of 1.2 ml. InGP, 4.5 μmoles, was included in substrate protection experiments. This reaction mixture was incubated in a water bath maintained at 27°-28° and irrigated with a 150-watt tungsten lamp at a distance of 15 cm. The reaction was terminated by placing the samples in the dark at 0° and adding 10 mg of acid-washed Norit A to remove the methylene blue. After filtration, the contents of each tube were then dialyzed overnight in the dark against 5 mM potassium phosphate buffer (pH 7.0). Three samples at each incubation time were obtained, viz. samples irradiated in the presence and absence of substrate and a nonirradiated control. Aliquots of each sample were assayed for enzyme activity. B, the kinetics of amino acid residue loss as a result of photo-oxidation in the presence and absence of InGP. The reaction conditions were identical with those described in A. Amino acid analyses after acid and alkaline hydrolysis (21) were performed on aliquots of the same samples in which enzyme activity was estimated.
experiment was performed before the results of EM and iodoacetate labeling were known; at that time cysteine was determined by the extent of EM-14C labeling. In view of subsequent knowledge that EM can react with only a net of one cysteine residue, the use of this method was inadequate as an assay of the cysteine content of this enzyme. For this reason, data on the loss of cysteine, which by this method was substantial nonetheless, are not presented in this experiment. It can be seen in Fig. 7B that methionine and histidine residues are oxidized to an appreciable extent whereas tyrosine was not. It should also be noted that the rates of histidine and methionine loss are approximately twice the rate of enzyme inactivation, a net of about 1.5 residues of each being oxidized after 60 minutes. In the presence of substrate, there is a net loss of approximately 0.5 residue each of histidine and methionine. There was virtually no loss of any amino acid residue in control samples incubated in the dark during the same period of time.

Because the method of EM-14C labeling of cysteine residues was unsatisfactory for the estimation of the decrease in cysteine content during photo-oxidation, the experiment was repeated and assays for cysteine by the iodoacetate method were performed (Fig. 8). It is seen that the rate of loss of cysteine (upper curve) also was approximately twice the rate of enzyme inactivation (lower curve). There was no decrease in activity nor cysteine content in samples incubated in the dark.

**Effect of Urea**—The presence of increasing concentrations of urea during the normal assay of the A protein results in a progressive decrease in activity, essentially complete inactivation occurring at 5 to 6 M urea (Fig. 9). Previously, it was shown that a maximum of 1 mole of EM was fixed per mole of enzyme (Fig. 2) and that the presence of urea resulted in no additional labeling of the protein by EM. Moreover, the presence of indole and 3-phosphoglyceraldehyde had also been shown to prevent labeling with EM (Fig. 3). However, protection experiments performed in the presence of 6 M urea indicated that indole and 3-phosphoglyceraldehyde could not prevent EM labeling.

**Effect of Photo-oxidation Urea (CM)**

The reaction conditions were identical with those described in Fig. 7.

**FIG. 8 (left). The kinetics of activity loss and cysteine loss as a result of photo-oxidation.**

**FIG. 9 (right). The effect of urea on enzyme activity.**

**DISCUSSION**

In view of the probable flexibility of enzyme structure in solution and the possibility that low molecular weight compounds, such as an enzyme substrate, alter the conformation of the protein, it becomes exceedingly difficult to define clearly an unambiguous role for a particular amino acid residue in which modification results in the loss of enzyme activity. For this reason, much emphasis in this initial investigation has been placed on the difference in the resulting chemical modification of the enzyme in the presence and absence of the substrate. A difference would certainly be observed which could more accurately reveal some specific characteristic of the functioning enzyme molecule. Nevertheless, it should be recognized at the outset of such a discussion that, depending on the extent to which substrate induces conformational changes, an observed difference in reactivity of a certain amino acid residue in the presence of substrate could be merely the result of a reduced accessibility of that residue quite distant and unrelated to the region of substrate binding and turnover. The problem is somewhat simplified with the enzyme examined here. First, there is but one substrate, InGP, and multiple effects possible in a situation in which there are two or more reactants are reduced. Second, the A protein is composed of a single polypeptide chain in contrast to proteins with a subunit structure involving a number of protein chains in which conformational considerations are compounded.

**Role of Cysteine Residues**—Two lines of evidence strongly indicate the requirement for one or more of the three cysteine residues as free sulfhydryls in the catalytic activity of the A protein. The first of these comes from experiments with sulfhydryl-binding reagents in which some unusual properties of the sulfhydryl groups are seen. It should be stated at this point that without doubt the freshly isolated enzyme molecule contains three free sulfhydryl groups (i.e. cysteine residues). The inability to detect these as such in the past was a result of the particular manner in which they were assayed.

It is difficult, at the present time, to propose a model for the
relative conformation of these side chains and for their contribution to enzyme activity which explains all of the results that have been presented. It does appear, however, from protection experiments, that a conformation exists in which the three sulfhydryl groups are arranged close to each other spatially, at least in the presence of EM, iodoacetate, or InGP. Moreover, the conformation assumed probably results in the positioning of the polypeptide chain, such that these residues are near the site of InGP binding or turnover. Evidence suggesting a difference between the site of InGP binding on the polypeptide chain and a site of substrate turnover will be discussed later.

The difference in labeling with iodoacetate or EM could be the result of the relative size of these reagents. Thus, reaction of EM may take place with any of the sulfhydryl groups, but once a molecule of EM had reacted, it could spatially block the other cysteine residues, preventing another molecule of EM from reacting. The protein must be quite rigid in the sulfhydryl region in view of the ineffectiveness of such denaturants as urea or guanidine to render all the sulfhydryl groups accessible to EM. Moreover, the presence of InGP blocks the reaction of any of the sulfhydryl groups with the relatively bulky EM moleucole. As a consequence of this relationship, it would appear that, when there is 1 mole of EM per mole of enzyme, the entrance of InGP is prevented; hence, enzyme activity is prevented and, conversely, the presence of InGP blocks the reaction of EM with any of the sulfhydryl groups and results in the preservation of activity. The phenomenon of EM labeling of any, and only one, of the cysteine residues is not unique. The reactivity of these sulfhydryl groups toward EM is quite analogous to the reactivity of two histidine residues in ribonuclease toward iodoacetate (22, 23). In that case also, carboxymethylation can occur with either of the histidine residues, but if one does react the other one cannot.

The reaction of the cysteine residues with iodoacetate, on the other hand, is distinguished by the fact that all three sulfhydryl groups can be substituted. This could be the result of the smaller size of the carboxymethyl group. The presence of InGP, however, allows only two molecules of iodoacetate to react with the sulfhydryl groups, and the relative arrangement of the three cysteine residues with respect to the substrate results in the protection by InGP of any one of them. This is consistent with the observation of complete enzyme inactivation in the absence of substrate only after 2 moles of carboxymethylcysteine have been formed. Thus, after one cysteine residue has been substituted, there may be some difficulty with InGP binding on the enzyme and partial inhibition results. At the point of formation of two carboxymethylcysteine residues, the substrate might not react with the enzyme at all and complete inhibition is observed.

One striking anomaly, however, does exist. This comes from a comparison of the activity observed when the enzyme is labeled with iodoacetate in the presence of InGP to that obtained after iodoacetate treatment in the absence of substrate. It can be seen by comparing the results shown in Fig. 4, A and B, that the enzyme, labeled to the extent of 1 mole of carboxymethylcysteine per mole of enzyme in the absence of InGP, is 30% inactivated. However, when reaction with iodoacetate occurs to the same extent in the presence of substrate, the enzyme is only about 20% inactivated. These two preparations of carboxymethyl enzyme must therefore be substantially different, although labeled to the same extent. As yet, there is no evidence suggesting a satisfactory explanation for this observation.

Additional support for the role of sulfhydryl groups in enzyme action comes from observations on the relative rates of enzyme inactivation and of amino acid residue loss during photo-oxidation. It is difficult with the existing data to relate enzyme inactivation unequivocally to the oxidation of a single amino acid residue. It was observed that the rate of oxidation of each amino acid residue (cysteine, histidine, and methionine) was approximately twice that of enzyme inactivation; e.g. at 50% inhibition of activity, 1.07, 0.88, and 0.98 residues of cysteine, histidine, and methionine, respectively, were oxidized. However, the relative rates of cysteine oxidation and enzyme inactivation were very similar to those observed with iodoacetate. In other words, the enzyme is completely inactive only when two sulfhydryl groups have been destroyed. For this reason, it is suggested that the major factor in enzyme inactivation by photo-oxidation is the oxidation of two cysteine residues. However, the possibility that activity loss is a result of photo-oxidation of the three types or any two types of amino acid residue, each contributing to a degree of activity, cannot be eliminated completely.

The exact mechanism whereby cysteine oxidation would inactivate the enzyme is unclear. Well, Gordon, and Buchert (24) have indicated that as a result of photo-oxidation, cysteine is oxidized beyond the cysteic acid state. It is pointless to suggest a mechanism until the identity of such a derivative is known.

Roles of Histidine and Methionine Residues—A number of observations have suggested that there are amino acid residues on the polypeptide chain involved in substrate binding which are distinct from the sulfhydryl groups. The initial indication for this suggestion comes from experiments with urea. The enzyme is completely inactive in 6 M urea although the reactivity of the cysteine residues with EM appears to be unchanged. Moreover, the presence of indole and 3-phosphoglyceraldehyde, which normally prevents the EM reaction, has no effect in the presence of urea. One explanation for these observations could be that in the presence of urea the substrate cannot approach the sulfhydryl group region and no activity results, although some features of the immediate conformation about this site is retained. An additional portion of the protein would appear to be required for substrate binding. There is somewhat stronger evidence for such a site on the A protein. As a result of the conclusions regarding the role of cysteine during inactivation by photo-oxidation, it becomes necessary to interpret the loss of histidine and methionine as not contributing substantially to enzyme inactivation. However, their presence near a site of substrate binding may be inferred from the fact that there is substrate protection of a net of one residue of each. The possibility cannot be excluded, however, as mentioned previously, that InGP induces a conformational change which makes these residues less accessible to photo-oxidation, when in fact they may reside elsewhere in the protein.

Corroborative evidence is available with regard to the possible role of histidine in substrate binding. This evidence comes from the observed inflection point of pH 5 to 6 in the plot of pKm.
versus pH (Fig. 1). If these data do indeed implicate a histidine residue in substrate binding, one is left to conclude either that the histidine residue which is photo-oxidized is different from the one involved in substrate binding or that photo-oxidation does not destroy the responsible ionic properties of the relevant histidine residue. Since it is thought that photo-oxidation of histidine involves a rupture of the imidazole ring (24), it is difficult to understand that such a process would not destroy its ability to act in the binding of InGP. The possibility should be kept in mind that the $K_m$ data may simply reflect the dissociation of the phosphate group in the substrate and not that of a protein side chain. The ionization properties of InGP are not known; however, the pK for glycerol phosphate falls between pH 6 and 7. This is slightly higher than was observed in our experiments. It is interesting to note that two of the cysteine-containing peptides, TP-29 and TP-25, contain, respectively, a histidine and methionine residue. Whether these residues are, in fact, those protected by substrate during photo-oxidation remains to be determined.

Of the amino acid residues believed to be essential or involved in some manner in the catalytic activity or substrate binding properties of the A protein (viz. cysteine, histidine, and methionine), none has been found to be altered in mutationally altered and enzymatically defective A proteins. It is, however, not unlikley, that the amino acid substitutions found in these defective proteins are important for proper folding of the normal proteins. In view of the suggested relative orientation of the sulfhydryl groups and possibly a histidine and methionine residue in the normal A protein, it is reasonable to expect that these altered A proteins would exhibit marked anomalies with respect to labeling by EM and iodoacetate or in response to photo-oxidation in the presence and absence of InGP. These studies are currently in progress.

SUMMARY

As a result of kinetic studies and studies on the chemical modifications of the A protein by photo-oxidation and by sulfhydryl group reagents, it was concluded that at least two of the three cysteine residues are required for enzyme activity.

$N$-Ethylmaleimide and iodoacetate labeling of the A protein has also indicated an unusual conformation of the polypeptide chain in the sulfhydryl group region.

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REFERENCES

Studies on the Active Site of the A Protein Subunit of the \textit{Escherichia coli} Tryptophan Synthetase

John K. Hardman and Charles Yanofsky