Substrate Binding Properties of Mutant and Wild-Type A Proteins of Escherichia coli Tryptophan Synthetase

Abstract. *Most of the mutant A proteins studied appear to be similar to the normal enzyme both in their apparent conformation about the critical cysteine residues and their ability to bind substrate. Two mutant proteins, in which a glutamic acid or arginine residue is substituted for a glycine residue, do appear abnormal suggesting that these primary structural changes radically affect the conformation in regions at or near the site or sites of substrate binding.*

Certain mutations in the A gene of the tryptophan operon of *Escherichia coli* result in the formation of altered A proteins which are enzymatically defective in catalyzing the conversion of indole-glycerol phosphate (InGP) to indole and 3-phosphoglyceraldehyde. A large number of such mutant A proteins have been isolated and found to have single amino acid differences from the wild-type enzyme (*1*). Other mutants are known whose mutationally altered sites are located at points throughout the A gene and produce altered A proteins which presumably have single amino acid changes at corresponding positions throughout their respective A proteins. The subject of this communication is the following question. How do these single alterations in primary structure result in enzyme inactivation? Specifically, do these changes result in loss of substrate-binding ability, or do they affect the active-site region in some other way?

An experimental approach to this problem presented itself when chemical modification of the wild-type protein was studied (*2*). Reaction of *N*-ethylmaleimide (NEM) with the enzyme resulted in the uptake of only 1 mole of this reagent and this uptake occurred concomitantly with complete inactivation. The presence of substrate, InGP, provided essentially complete protection against NEM substitution and enzyme inactivation. Moreover, from the manner in which NEM reacted with the enzyme, an unusual conformation in the sulfhydryl-group region was revealed. Although only 1 mole of NEM could be bound to the protein, each of the three cysteine residues present reacted equally with the reagent and in such a manner that substitution of NEM on any one prevented reaction with the remaining two cysteine residues.

There exist, therefore, several readily measurable characteristics of the wild-type A protein which are apparently associated with its catalytic activity. Of importance for the present investigation is the fact that these properties can also be studied with A proteins which are enzymatically inactive. Thus, each mutant protein could be compared to the normal enzyme with respect to (i) the conformation about the cysteine residues, as determined by the increase or decrease in the level of NEM labeling, and (ii) its ability to bind substrate as determined by the extent of protection by InGP against NEM labeling.

Figure 1 shows a representation of the normal A protein with the approximate locations of the cysteine residues which are protected by substrate, and the sites of amino acid changes in the mutant proteins studied as established by analysis or deduced from the locations of genetic alterations on the map of the A gene (*3*). It can be seen that, in the mutants examined, these changes occur at different points in the protein and involve residue changes of the types: uncharged to uncharged, uncharged to charged, and charged to uncharged.

The results of NEM labeling experiments with several of the mutant A proteins are presented in Fig. 2. In the absence of substrate and under the conditions employed, there is no striking difference between the NEM labeling patterns of the normal protein and the A58, A34, A89, and A85 mutant proteins. The A3 preparation does show a slightly lower level of NEM bound per mole of protein. The fact that the A3 preparation used was slightly impure and contained 75 to 80 percent A protein may account for the decreased NEM substitution. This suggestion is supported by the observation that the extent of NEM labeling in the presence of InGP is also slightly lower than with

Table 1. *N*-Ethylmaleimide (NEM) and iodocacetate labeling of wild-type and A78 A proteins.

Conditions of labeling	NEM bound (mole/mole of protein)		Iodoacetate bound (mole/mole of protein)	
	Wild type	A78	Wild type	A78
Without InGP	1.00	1.75	3.00	3.75
With InGP ($6.5 \times 10^{-3}M$)	0.15	1.20	2.00	2.60

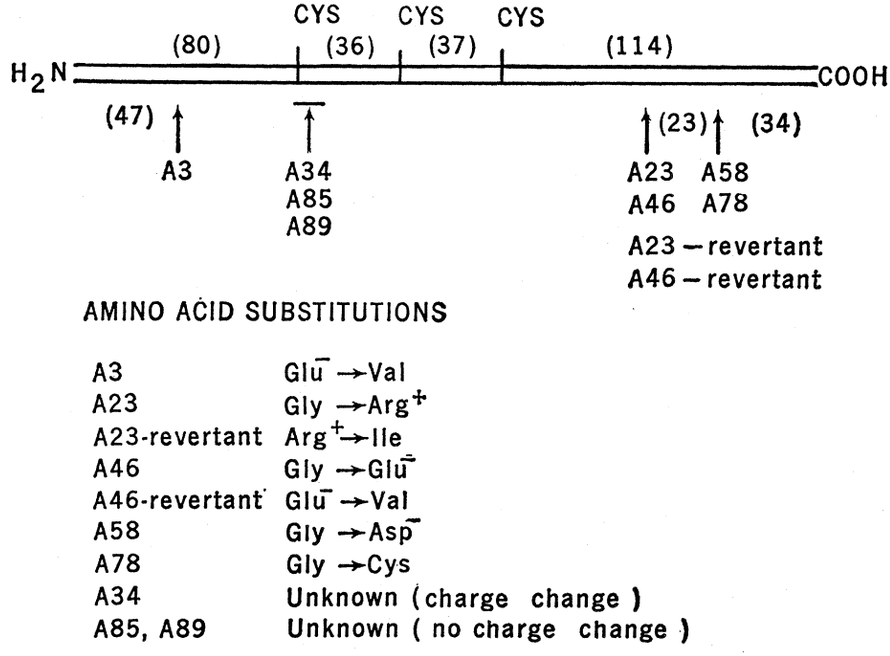

Fig. 1. Representation of the A protein. The sites of amino acid substitution in the various mutant proteins are indicated by the arrows. The sites of the pertinent cysteine residues are indicated above the line. In the parentheses, the residue distances between the sites are given. The insert shows the known amino acid substitutions in the mutant and revertant proteins.

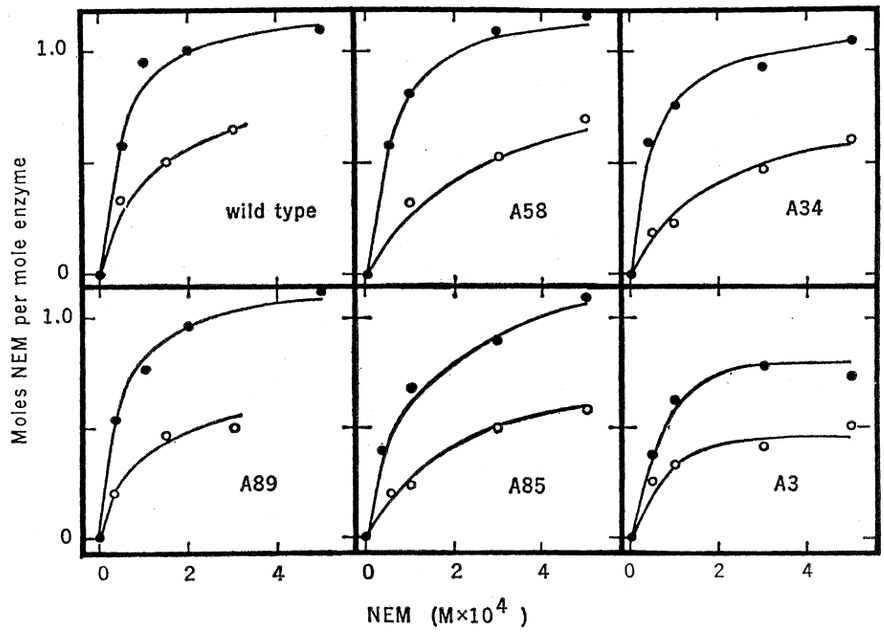

Fig. 2. The NEM labeling patterns of wild-type and mutant A proteins in the absence (solid circles) and presence (open circles) of 4 × $10^{-3}M$ InGP.

the wild-type protein, although the percentage of protection in each case is nearly identical (see below). These proteins were also examined for NEM labeling in the presence of substrate. To obtain a quantitative measure of substrate binding ability, a level of InGP was chosen which provided about 50 percent protection with the wild-type enzyme (lower curve in each frame of Fig. 2). With each of these mutant proteins, substrate protected against NEM labeling. From these data, it is possible to compare substrate protection of mutant and wild-type proteins quantitatively by a simple calculation of the difference in the extent of NEM labeling in the presence and absence of InGP in each case. When this is done, the extent of protection over the range of NEM concentrations used was virtually identical for the normal and mutant proteins.

Mutant A78, which contains an inactive A protein with a glycine-to-cysteine change 35 residues from the carboxyl terminus (Fig. 1), was thought to represent a somewhat special case. Since this protein, when oxidized with performic acid, contains four cysteic acid residues, an obvious explanation for the lack of enzymatic activity would be the formation of a disulfide bond between the extra cysteine residue and one of the cysteine residues in the critical sulfhydryl-group region. The data in Table 1 indicate that this is probably not the case. Under normal conditions of NEM substitution, 1.75 to 1.8 moles of NEM are bound per mole of the A78 protein. This is in contrast to the maximum of 1 mole of NEM bound to the wild-type enzyme. Moreover, in the presence of a saturating amount of InGP, which provides about 85 percent protection against labeling of the maximum of 1 mole of NEM with the wild-type enzyme, about 1.2 moles of NEM are bound to the A78 protein. These results would be expected if there were simply an additional free sulfhydryl group in the A78 protein. Results obtained in experiments designed to examine iodoacetate labeling of the cysteine residues tend to confirm this conclusion. Reaction of the cysteine residues in the normal enzyme with iodoacetate differs from the reaction with NEM, in that 3 moles of the reagent are bound per mole of enzyme. InGP will prevent the binding of a net of 1 mole of iodoacetate (*2*). Under the conditions employed no other residues in the wild-type protein react with iodoacetate. With the A78 protein, however, 3.75 and 2.60 moles of iodoacetate are bound in the absence and presence of InGP, respectively (Table 1). This finding also suggests that InGP can prevent the binding of a net of approximately 1 mole of the reagent.

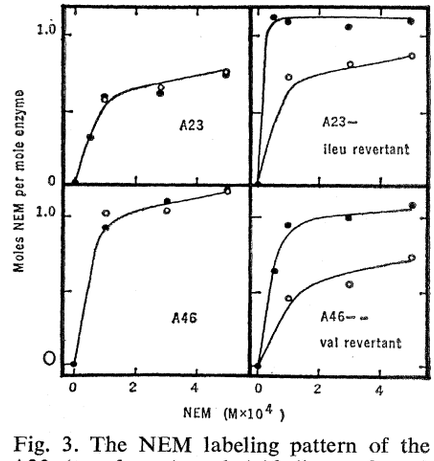

Fig. 3. The NEM labeling pattern of the A23 (top frame) and A46 (lower frame) proteins in the absence (solid circles) and presence (open circles) of 4.3 × $10^{-3}M$ InGP. The NEM labeling pattern of the "A23 ileu-revertant" (top frame) and "A46 val-revertant" (lower frame) proteins in the absence (solid circles) and presence (open circles) of 4.3 × $10^{-3}M$ InGP. The "A23 ileu-revertant" and "A46 val-revertant" proteins are described in the text.

The A proteins from two additional mutants, A46 and A23, have been examined and appear to be substantially different in behavior from those mentioned (Fig. 3). With the A46 protein, there is still approximately 1 mole of NEM bound per mole of protein, but here, in the presence of InGP, there is no protection. The A23 protein represents a slightly different situation in that not only is there a complete lack of protection by substrate, but also the extent of NEM labeling appears to be somewhat reduced. The A proteins of partial revertants of each of these mutants were also examined. These proteins have slight enzymatic activity, and it was of interest to determine whether substrate protection was regained. In one of the proteins a valine residue is substituted for the mutant glutamic acid residue in the A protein of A46. In the other, isoleucine replaces the arginine residue introduced by the A23 mutation. It can be seen (Fig. 3) that wild-type behavior is restored with respect both to the ability of InGP to protect and, in the case of the A23 protein, to the extent to which NEM labels the protein.

Chemical modification studies with a variety of enzymes have established that alteration of single amino acid residues can result in inactivity. Mutations to resistance to inhibition by compounds which are analogs of enzyme substrates are associated with enzyme changes that alter the affinity for the analog (*4*). Similarly, mutant proteins have been detected which are enzymatically defective as a result of a decreased capacity to bind a required ion (*5*). These and similar cases convincingly demonstrate that small, local changes in en-

zyme structure can have profound effects on the ability of an enzyme to function effectively.

The A3, A34, A58, A78, A85, and A89 proteins appear to have retained both the unusual wild-type conformation in the sulfhydryl-group region as well as the ability to bind substrate. It should be pointed out, however, that these treated proteins were not further characterized to determine whether NEM had reacted with the cysteine residues in the same fashion as in the normal enzyme, although this does seem likely.

Ignoring this reservation one would conclude that alteration of some property other than the conformation in the cysteine-containing region and the ability to bind substrate is responsible for their lack of enzymatic activity or that the methods used are not sufficiently sensitive to detect subtle, yet critical, changes. There is the possibility, for example, that these methods cannot detect "proper" substrate binding, which may involve specific and additional associations required for substrate turnover that are not possible with the mutant proteins.

The mutant A proteins from A46 and A23, on the other hand, do show marked differences in behavior from the wild-type enzyme. Both mutant proteins appear to lack the ability to bind InGP at the cysteine-containing region. Although these proteins do have charge differences from the normal enzyme (Fig. 1), this fact alone could not explain their behavior since three of the other mutant proteins, A3, A34, and A58, also have charge changes and are similar to the normal enzyme in substrate-binding properties. However, the A46-A23 site in the protein may be more critically involved in maintaining a proper conformation for InGP binding than some other regions of the polypeptide chain and thus a charge change here may have a more severe effect. The finding with A46 and A23 revertant proteins (containing either a valine or isoleucine residue in place of a charged residue) that there is concomitant restoration of enzymatic activity and substrate-binding ability supports this view.

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References and Notes

1. D. R. Helinski and C. Yanofsky, *Proc. Nat. Acad. Sci. U.S.* **48**, 173 (1962); U. Henning and C. Yanofsky, *ibid.* pp. 183 and 497; D. R. Helinski and C. Yanofsky, *J. Biol. Chem.* **238**, 1043 (1963); J. R. Guest and C. Yanofsky, *ibid.* **240**, 679 (1965); B. C. Carlton and C. Yanofsky, *ibid.*, p. 690.
2. J. K. Hardman and C. Yanofsky, *J. Biol. Chem.* **240**, 725 (1965).
3. C. Yanofsky, G. R. Drapeau, J. R. Guest, B. C. Carlton, *Proc. Nat. Acad. Sci. U.S.* **57**, 296 (1967).
4. R. D. Hotchkiss and A. H. Evans, *Fed. Proc.* **19**, 912 (1960); A. M. Albrecht, J. L. Palmer, D. J. Hutchinson, *J. Biol. Chem.* **241**, 1043 (1966).
5. M. J. Schlesinger, *J. Biol. Chem.* **241**, 3181 (1966).
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Yeast Transfer RNA: A Small-Angle X-Ray Study

Abstract. *The intensity of x-ray scattering and the radius of gyration were measured for a mixture of yeast transfer RNA's in tenth molar potassium chloride. The experimentally observed radius of gyration eliminates single-stranded, hairpin, singly folded hairpin, triple-stranded, and linked double-hairpin models of tRNA but allows certain folded cloverleaf models. The measured intensities at larger angles, beyond the radius of gyration region, lend some support to the Holley's cloverleaf model, in which three arms are folded up tightly together and the fourth arm is extended in the opposite direction.*

The base sequences are known (*1*, *2*) for several transfer RNA's (tRNA) from yeast, and there have been a number of suggestions of possible base pairings and molecular conformations. However, the molecule has not been crystallized, and the tertiary structure is not known. We have measured the x-ray scattering from dilute solutions of tRNA and have calculated the scattering expected from several arrangements of Holley's (*1*) cloverleaf model of the molecule. Most of the more open or extended tertiary structures are unlikely.

Measurements of x-ray scattering at small angles were made upon a mixture of yeast transfer RNA's in 0.1*M* KCl, 0.02*M* tris (hydroxymethyl) aminomethane-hydrochloride buffer at *p*H 7.42. The x-ray data were corrected simultaneously for effects due to slit height and width (*3*). The radius of gyration, *Rg*, was found to be 23.5 Å ± .25 Å, and the weight-average molecular weight, as determined from the absolute forward scattering, was 27,500. The x-ray scattered intensities in the shape region (that is, the region from 10 to 70 milliradians for tRNA, see Fig. 2) do not agree well with the scattering calculated from simple shapes, such as ellipsoids of revolution and cylinders. Radii of gyration for mixtures of yeast tRNA have been published by Krigbaum and Godwin (*4*) (Rg = 23.9 Å) and Dembo, Sosfenov, and Feigin (*5*) (Rg = 21 Å), both values being in reasonable agreement with ours.

The tRNA was prepared by a slight modification of Holley's method (*6*). After chromatographic fractionation of the tRNA (*7*), it was found that the mixture consisted mainly of six tRNA's and included alanine tRNA. The tRNA was then precipitated with ethanol and dried at 4°C over phosphorus pentoxide. At the end of 2 days it was dissolved in 0.1*M* KCl containing 0.02*M* tris-HCl, at *p*H 7.42, and a small amount was chromatographed on Sephadex G-100. The column gave a sharp, symmetrical peak without evidence of dimer or degradation products.

The most striking experimental result is the small radius of gyration. The radius of gyration is measured from the curve of scattered x-ray intensity in the region from 8 to 10 milliradians for tRNA (see Fig. 2) and is defined by

$$(Rg)^2 = (\Sigma\, n_i \cdot r_i^2)/(\Sigma\, n_i)$$

The number of electrons in the ith atom is n_i; r_i is the distance of the ith atom from the center of charge of the molecule, and the sum is over all atoms of the molecule. The experimental radius of gyration may be used to eliminate several tertiary structures which have been suggested. The calculated radius of gyration (28 to 30Å) of the random coil of a single-stranded model is too large. A hairpin model of the tRNA has a radius of gyration of about 40 Å and thus is certainly eliminated, while the same hairpin model folded back upon itself at the middle still has too large a radius of gyration (about 25 Å). A triple-stranded model (*8*) also has too large a radius of gyration (about 28 Å). The model consisting of two linked hairpin regions (*1*) has a radius of gyration of about 25 Å and is probably eliminated. Of the configurations suggested by others, only certain folded Holley cloverleaf models appear to have radii