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*AN ALTERATION IN THE PRIMARY STRUCTURE OF A PROTEIN PREDICTED ON THE BASIS OF GENETIC RECOMBINATION DATA**

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Studies on the fine structure relationships between a gene and the corresponding protein are being carried out with the A protein of the tryptophan synthetase of *Escherichia coli*. The preceding paper[1] describes an investigation with altered A proteins from several mutants and demonstrates that mutational changes at or near the same site in the A gene lead to alterations in the same region of the A protein.

Studies[2, 3] with a large number of A-protein mutants have shown that there are two closely linked sites at one end[4] of the A gene that have mutated frequently. All of the strains with mutational changes at one of these sites form a heat-labile A protein[2] that is distinguishable from the wild-type A protein in peptide pattern studies.[1] Strains with mutational changes at the second site form an altered A protein that is somewhat more heat-resistant than the wild-type A protein[2] but cannot be distinguished from the normal protein in peptide pattern studies.[1] The distance between the two mutational sites is approximately $^1/_{625}$–$^1/_{2500}$ of the total length of the map of the A gene.

Since the A protein appears to be a single polypeptide chain[5] containing approximately 280 amino acids,[6] one would predict that these two mutant types should have amino acid substitutions at or near the same position in the A protein if the number of nucleotide pairs in the A gene is a small multiple of the number of amino

acids in the A protein and if distances on the genetic map bear some relation to the distances between corresponding amino acids in the A protein. This report is concerned with an examination of this prediction.

Materials and Methods.—Mutants: The mutant strains mentioned in this paper were obtained by penicillin selection[7] following ultraviolet irradiation of wild-type strain K-12 of *E. coli*.

Isolation of peptides and amino acid analyses: Tryptic digests of the isolated A proteins were prepared as described previously.[8] The trypsin-resistant core of the A protein was removed from the digests by isoelectric precipitation at pH 4.[10] The soluble peptides were separated by the method of Rudloff and Braunitzer[11] with minor modifications in order to improve the separation of the A protein tryptic peptides. In principle, the procedure consists of column chromatography of the acid-soluble supernatant on Dowex 1 × 2 (acetate form) starting with a collidine acetate buffer at pH 8.5. A pH gradient with 0.09 *N* acetic acid was applied and then a second pH gradient was used with 1.5 *N* acetic acid. The peptide discussed in this communication, TP3, was eluted near the end of the first pH gradient. The TP3 obtained by Dowex chromatography often contained minor contaminants and was further purified by paper chromatography or paper electrophoresis in the solvent system or buffer, respectively, used in peptide pattern studies.[1, 8] Complications resulting from purification by paper chromatography are mentioned in Table 1. Acid hydrolysis of peptides was performed in evacuated, sealed tubes in 5.7 *N* HCl at 105°C for 48 hr. Amino acid analyses were carried out with a Spinco amino acid analyzer.

Results.—Characteristics of mutant A 46 and the altered A protein it produces: Tryptophan auxotroph A 46 is a typical A mutant of *E. coli* K-12.[12–14] It accumulates indoleglycerol and can grow on a minimal medium supplemented with tryptophan or indole but it will not respond to anthranilic acid. It forms an altered A protein that is fully active in the conversion of indole plus serine to tryptophan in combination with normal B protein but is inactive in the other two tryptophan synthetase reactions, the reversible conversion of indoleglycerol phosphate to indole and the conversion of indoleglycerol phosphate plus serine to tryptophan. The A-46 protein has the same affinity for normal B protein as the wild-type A protein and is slightly more heat-resistant than wild-type A.[2] It protects wild-type A protein from neutralization by antibody to the wild-type A protein and appears to react with the antibody to the same extent as the normal A protein.[2]

The isolated A 46 protein was examined electrophoretically under various conditions to determine if there was a charge difference from the normal A protein. Different mobilities were observed on cellulose acetate (Fig. 1), at pH 9.2, indicating that the mutant protein has a higher negative net charge than the normal A protein. At pH 7.5, where the A protein is still negatively charged (the isoelectric point of the wild-type protein is at pH 5),[6] this charge difference could not be demonstrated.

The wild-type A protein can be crystallized under certain conditions from a 40–45 per cent ammonium sulfate solution.[6] Repeated attempts to crystallize the isolated A 46 protein, however, were unsuccessful. Instead of crystallizing, the originally slightly turbid protein solution congealed and after several days assumed a consistency similar to that of soft agar. The gel dissolved readily in water.

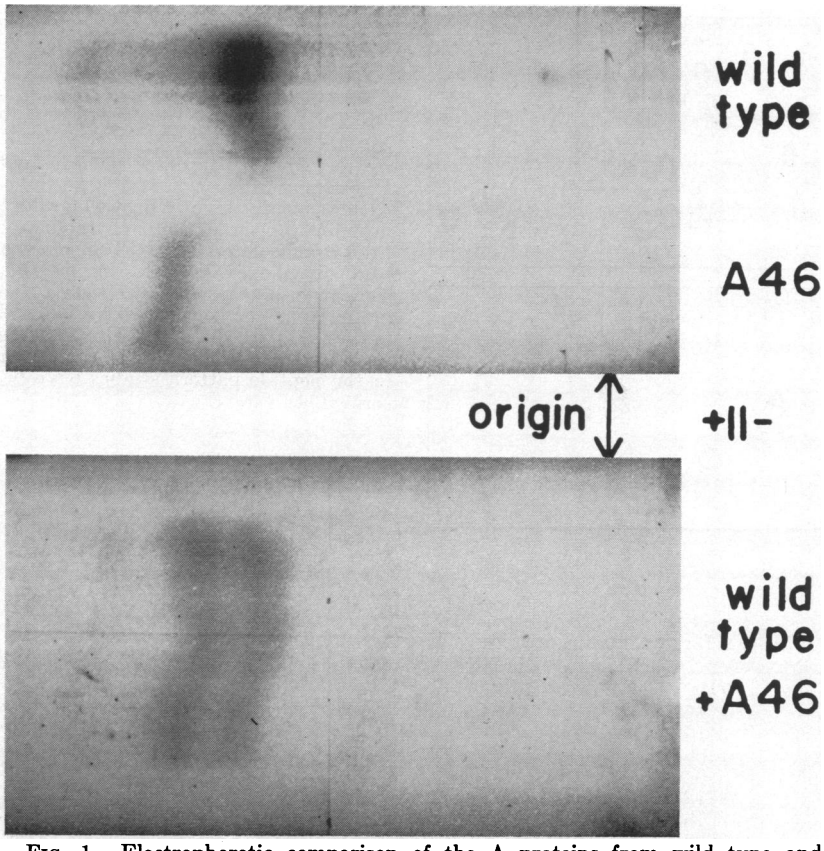

FIG. 1.—Electrophoretic comparison of the A proteins from wild type and mutant A 46. Approximately 30 μg of each purified protein was applied to cellulose acetate strips (5 × 10 cm.). Electrophoresis was performed in 0.04 *M* sodium diethylbarbiturate buffer, pH 9.2, at 4°C for 11 hr at 36 V/cm (3 mA). Ponceau S stain.[24]

Microscopical examination of the gel did not reveal any organized structure.

Examination of the wild-type and A 46 tryptic peptides corresponding to the tryptic peptide altered in mutant A 23: The amino acid substitution in mutant A 23 described in the preceding paper[1] occurs in a region of the A protein that corresponds to the wild-type tryptic peptide TP3. In spite of the absence of any differences in trypsin or trypsin plus chymotrypsin peptide patterns of the A-46 protein and the normal A, the fact that the genetic alterations in mutants A-23 and A-46 map close to one another prompted an examination of peptide TP3 from the A protein of wild type and A-46. The location of this peptide on fingerprints of tryptic digests of the A protein is shown in Figure 2. Peptide TP3 was isolated from the wild-type and A-46 proteins as described in the *Methods* section. The amino acid composition of this peptide from the two sources (Table 1) clearly showed that the mutant peptide contained a glutamic acid or glutamine in place of a glycine. Since wild-type TP3 contains two glycine residues, it was necessary to degrade the peptide further to determine which glycine was affected by the mutation. Chymotrypsin treatment of TP3 resulted in the liberation of three peptides designated TP3C1, TP3C2, and TP3C3 (Fig. 2). Fortunately, the glycine residues proved to be in different

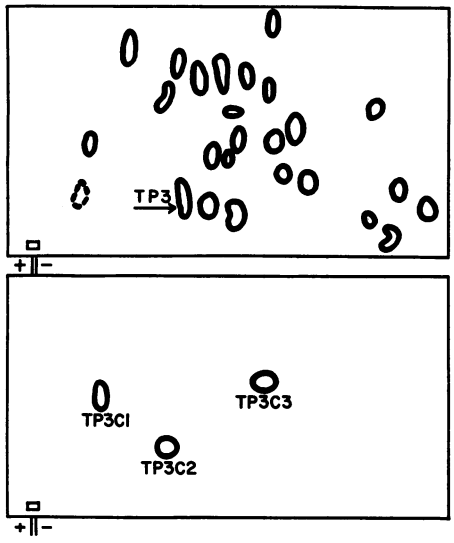

FIG. 2.—Tracings of peptide patterns. Upper figure: location of TP3 in the peptide pattern from a tryptic digest of wild-type A protein. Lower figure: chymotryptic peptides from isolated TP3. The same patterns were obtained with the corresponding peptides from mutant A-46. Origin: □; chromatography in vertical direction; electrophoresis, at pH 3.7, in horizontal direction; ninhydrin stain. The conditions employed in the peptide pattern studies have been described.[1,8]

TABLE 1

MOLAR RATIOS* OF CONSTITUENT AMINO ACIDS IN CORRESPONDING PEPTIDES

Amino acid	Wild type peptides				Mutant A 46 peptides				Mutant A 95 peptides			
	TP3†	TP3C1	TP3C2	TP3C3	TP3	TP3C1	TP3C2	TP3C3	TP3	TP3C1	TP3C2	TP3C3
Lysine	1.87		1.11	0.94	1.85		1.00	1.00	2.05		0.95	1.06
Aspartic	2.13	0.98	1.13		1.98	0.94	1.03		1.97	0.94	1.00	
Serine‡	1.09		1.10		1.16		0.97		0.93		1.07	
Glutamic	3.04	1.11	1.05	1.16	4.12	2.11	1.15	1.21	4.15	2.13	0.99	1.17
Proline	3.12	2.08	1.03		2.84	1.95	1.05		2.87	1.93	1.17	
Glycine	2.16	1.05	1.05		1.22		0.89		1.13		0.99	
Alanine	2.92	1.81	0.90		2.91	1.95	0.98		2.95	1.98	1.05	
Valine	1.07		1.05		0.94		0.93		1.05		0.92	
Isoleucine	1.00		0.83		1.01		0.92		0.96		0.90	
Leucine	1.89	1.07		1.04	1.88	1.08		0.84	2.08	1.08		0.98
Tyrosine	0.91			0.87	—**			0.95	0.83			0.88
Phenylalanine	0.96	0.92			0.93	0.96			1.02	0.92		
Ammonia§	4.24				4.19				4.27			

* Calculation of molar ratios according to Hirs, Moore, and Stein.[26]
† Hydrolysis for 72 hr did not change these molar ratios.
‡ Corrected for partial destruction upon acid hydrolysis.
§ Not extrapolated to zero time hydrolysis.
** Complete or partial loss of tyrosine upon acid hydrolysis was observed when the peptide was purified by paper chromatography. That the tyrosine is still unaltered after the chromatography can be seen from the analysis of TP3C3 which was derived from this TP3 and was purified by paper electrophoresis. The product of the degradation of tyrosine has been found but not yet characterized.

peptides (Table 1) and it was immediately evident that TP3C1 contained the alteration. TP3C1 isolated from mutant A-23 does not contain glycine but has one arginine residue;[1] thus, the same glycine residue is replaced in the two mutant proteins.

It was concluded from paper electrophoretic studies that the amino acid substitution in A-46 involved glutamic acid and not glutamine. Since the γ-carboxyl group of glutamic acid has a $pk'_2 = 4.25$,[15] it would be expected that at higher pH values peptide TP3C1 from mutant A-46 would have an extra negative charge when compared to the wild-type peptide if the change involved glutamic acid. Electrophoresis of the peptides at pH 6.7 (Fig. 3) clearly demonstrated a charge difference. Consistent with this result are the ammonia values in the amino acid analyses of

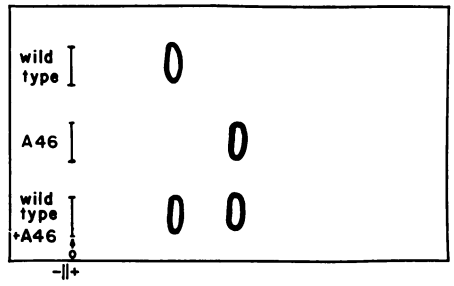

FIG. 3.—Electrophoresis of TP3C1 from the A proteins of wild type and mutant A-46 at pH 6.7. Approximately 0.06 μM of each peptide was applied to Whatman 3 MM paper, and electrophoresis was carried out in pyridine acetate buffer, pH 6.7,[25] at 55 V/cm (160 mA) for two hr at 20°C; ninhydrin stain.

TP3 from the wild-type and A-46 proteins which do not differ from each other (Table 1) and the greater electrophoretic mobility of the A-46 protein at pH 9.2 (Fig. 1). In the standard fingerprinting technique employed,[8, 9] electrophoresis is performed at pH 3.7. At this pH, the γ-carboxyl group of the glutamic acid residue evidently is not sufficiently dissociated to affect the migration of the peptide.

Examination of mutant A-95: Mutant A-95, an A mutant isolated recently, forms an altered A protein with properties identical to those of the A-46 protein. Genetic tests failed to detect recombination between mutants A-95 and A-46, while both gave identical recombination values with A mutants that map at other sites in the A gene. These findings suggest that A-46 and A-95 represent repeat mutations at the same genetic site. If, in addition, the mutational alteration was identical in both mutants, the same amino acid substitution should be present at the same position in their A proteins.

Peptide TP3 was isolated from a tryptic digest of the A protein of mutant A-95 and was digested with chymotrypsin to obtain the three peptides TP3C1, TP3C2, and TP3C3. The amino acid composition of all four peptides was determined and is given in Table 1. Since the same change observed in mutant A-46 was found in the A-95 protein, it is probable that the mutational alterations in these two mutants are identical. Three other A mutants, A-61, A-76, and A-86, which are also indistinguishable from A-46 in all tests performed to date, may also have the same amino acid substitution that was detected in A-46; the A proteins produced by these mutants have not yet been examined for the amino acid substitution.

Tentative structure of the tryptic peptide TP3: The result of chymotryptic treatment of TP3 agrees with expectations based on the usual specificity of chymotrypsin, since one tyrosine peptide and one phenylalanine peptide are liberated. TP3C2 does not have an aromatic amino acid and, therefore, is probably the C-terminal chymotryptic peptide of TP3. Furthermore, since TP3 is a tryptic peptide that contains two lysine residues, lysine is probably the N-terminal amino acid and TP3C2 is probably the N-terminal chymotryptic peptide of TP3. Several cases have been described[16, 17] in which an N-terminal basic amino acid is not liberated by trypsin from a peptide.[18] Other evidence suggesting the order TP3C3–TP3C1–TP3C2 for the chymotryptic peptides is the finding that the phenylalanine of TP3C1 is the N-terminal amino acid of TP3C2 obtained from trypsin plus chymotrypsin digests of the A protein from mutant A-23.[1] The phenylalanine is in the N-terminal position of TP3C2 from this mutant protein, since trypsin hydrolysis of the arginyl-phenylalanine bond leaves the phenylalanine N-terminal and thus resistant to release by chymotrypsin.[21] Consistent with this order are observa-

tions on the ninhydrin staining reactions of the various peptides. Peptides TP3, TP3C2, and TP3C3 stain blue while peptide TP3C1 stains yellow-brown; TP3 would be expected to stain yellow-brown if TP3C1 were its N-terminal chymotryptic peptide. These considerations plus the results of N-terminal amino acid sequence analyses of TP3C2[22] suggests the tentative structure of TP3 shown in Figure 4.

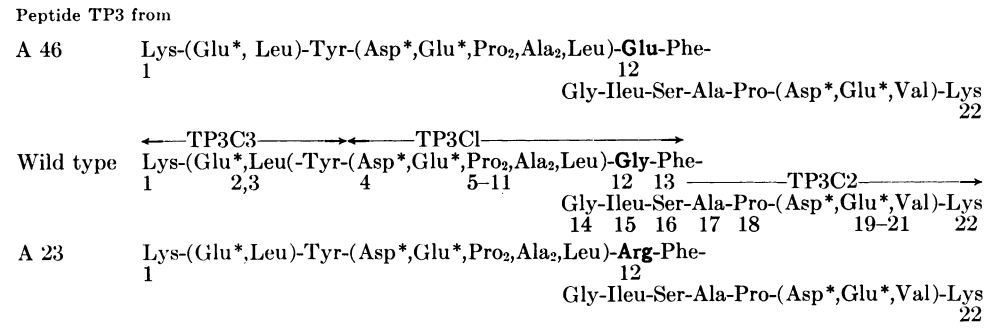

FIG. 4.—The amino acid substitutions in the tryptic peptide TP3 from mutants A-46 and A-23. Evidence for, and possible objections to, this representation are given in the text. Glu*, Asp*,: unknown whether present as amides. Ammonia values (Table 1) suggest the presence of four amide nitrogens in the peptide.

Discussion.—The results presented in this paper demonstrate a single amino acid substitution of a glycine by a glutamic acid as the result of mutation in strain A-46. The electrophoretic behavior of the isolated A-46 protein is consistent with this substitution, since a higher net negative charge than that of the wild-type protein is observed. The site of this switch appears to be identical with the site of the replacement of a glycine by an arginine in the A protein of mutant A-23[1] (Fig. 4). Although this is the simplest interpretation of the data, two objections to the representation shown in Figure 4 remain to be removed by sequence analysis of the amino acids constituting TP3C1. The A-46 protein could have undergone two amino acid changes: glycine replaced by glutamine and, if the other glutamic acid of the peptide were present as the amide, replacement of this amide by glutamic acid. Another possibility, also consistent with the results obtained, is that the sequence 5–12 (Fig. 4) in TP3 is not identical in the A-23 protein and wild-type A. The position of the glycine residue in the wild-type peptide TP3C1 was deduced from the position of the arginine in this peptide from mutant A-23 and thus could be incorrect if more drastic changes than a single amino acid substitution had occurred in the A protein of mutant A-23. Regardless of the amino acid sequences in TP3C1 from the wild-type protein and the proteins from mutants A-23 and A-46, the prediction of the location of the amino acid change in the A-46 protein, based on recombination data with mutant A-23, was found to be true.

The replacement of glycine by glutamic acid and arginine in the two mutants suggests that different nucleotide changes in the coding unit specifying the glycine in question have occurred. If the deduced amino acid sequence is correct, it can be further assumed that different nucleotides in the same coding unit were changed by mutation, since the two mutant strains can recombine to give wild-type progeny.

Evidently, the substitution of glycine by glutamic acid or arginine in a certain

position in the A protein causes the complete loss of enzymatic activity in the indoleglycerol phosphate–to–tryptophan and the indoleglycerol phosphate–to–indole reactions without affecting the immunological properties of the protein or the capacity to combine with normal B protein. The unaltered ability of these mutant A proteins to catalyze the indole-to-tryptophan reaction in combination with normal B supports the theory[14] that the A protein serves as an activator of the B protein in this reaction. Apparently, a region of the A protein unaffected by the amino acid changes in the mutant proteins is required for this function. One might also conclude that the glycine replaced in the A-46 and A-23 proteins is at, or affects, the active site of the protein. Replacement of this glycine by glutamic acid or arginine could alter the proper structure of this site so that it cannot be either maintained or formed in the sense of the "induced-fit" theory.[23] In view of the polarity of the residues introduced by mutation, this possibility is not difficult to visualize. It seems remarkable, in addition, that substitution of an uncharged residue by groups with opposite charges leads to opposite sensitivities to elevated temperatures. In this connection, it is also of interest that the presence of the extra negatively charged residue in the A protein of mutant A-46 leads to gel formation, which presumably is due to aggregation.

The studies with the A protein of mutant A-95 demonstrate that a mutational event classified as identical with that of mutant A-46 on the basis of genetic tests and comparisons of the properties of A proteins has, in fact, caused the identical amino acid substitution observed in mutant A 46. Since the A proteins of all five mutants that map at the A 46 site exhibit identical properties and since the A proteins of all nine mutants that map at the A-23 site also have identical properties, it would appear that the group of nucleotides coding for glycine in the normal A protein at the A-23–A-46 position is particularly susceptible to mutation following ultraviolet irradiation. Whether this susceptibility reflects a basic instability of this coding unit, a particular sensitivity to ultraviolet irradiation, or the possibility that only select classes of mutants forming certain types of altered A proteins can be recovered remains to be determined.

Summary.—The A protein of the tryptophan synthetase of mutant A-46 of *E. coli* K-12 was found to differ from the normal A protein by a single amino acid substitution, glutamic acid for glycine. The substitution results in alterations of several of the properties of the A protein. The glycine residue replaced by glutamic acid in the A-46 protein is the same glycine replaced by arginine in the A protein of the closely linked mutant, A-23.[1] The recombination observed between the two strains probably represents recombinational events between different nucleotides in the same coding unit.

Mutant A-95 and three other A mutants that map at the A-46 site form altered A proteins that exhibit the same properties as the A-46 protein. The A-95 protein was found to have the same amino acid substitution at the same position as the A-46 protein.

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† Fulbright grantee 1960–62.

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*EFFECTS OF FLUORODEOXYURIDINE ON DNA REPLICATION, CHROMOSOME BREAKAGE, AND REUNION**

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Fluorodeoxyuridine (5-fluorouracil deoxyriboside, FUDR) is phosphorylated in some cells. The fluorodeoxyuridylate formed is a highly active inhibitor of the enzyme, thymidylate synthetase;[1] therefore, the conversion of deoxyuridylate to thymidylate is blocked. Since this is the only pathway for the synthesis of thymidylate in most cells and their pool of thymidylate is usually very small, DNA replication is quickly blocked. The block can be overcome by supplying thymidine from an exogenous source. Since thymidylate functions primarily as a precursor