Proc. Natl. Acad. Sci. USA
Vol. 74, No. 1, pp. 286–290, January 1977
Genetics

Structure and properties of a hybrid tryptophan synthetase α chain produced by genetic exchange between *Escherichia coli* and *Salmonella typhimurium*

(hybrid polypeptide/intragenic recombination/molecular evolution)

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Contributed by Charles Yanofsky, October 1, 1976

ABSTRACT **Genetic exchange between the structural genes for the α chain of tryptophan synthetase [tryptophan synthase; L-serine hydro-lyase (adding indoleglycerol-phosphate), EC 4.2.1.20] of *E. coli* and *S. typhimurium* yielded recombinant genes that specified functional hybrid polypeptides. The α chains produced by three recombinants appeared to be identical but differed from those of *E. coli* and *S. typhimurium* by at least 27 and 8 amino acid residues, respectively. *In vivo* and *in vitro* tests of enzyme function suggest that the hybrid α chains are near-equivalent to their fully active parental proteins.**

Comparative studies of the amino acid sequences of homologous proteins of members of different taxonomic groupings have revealed extensive sequence variation (1, 2). This variation is apparently compatible with the identical or near-identical function of the homologous proteins in their respective organisms (3). The extent of amino acid sequence variation often increases with increasing periods of evolutionary divergence, allowing molecular confirmation of phylogenetic relationships established on the basis of classical criteria (4–6). Examinations of homologous proteins of individuals of present-day populations of the same species also reveal primary structure differences (7–10), indicating that multiple alleles coexist at many, if not most, loci. The two categories of variation mentioned presumably reflect stages in a continuing evolutionary process. Such variation challenges the biologist to deduce the basis of protein structure evolution.

Two views have been expressed concerning the significance of protein sequence variation. One view holds that the observed variation is due primarily to the balancing effect of a combination of selective forces (11–13). The other school suggests that most observed sequence variation reflects stages in the processes of fixation of selectively neutral or inconsequential amino acid changes or loss of deleterious changes (14–16). It is important that we learn which explanation is correct, since if it is the latter one, fundamental concepts of current evolutionary theory must be reexamined.

It occurred to us that one way of assessing the functional significance of the amino acid differences that have accumulated in homologous proteins of different species is to examine the characteristics of hybrid proteins produced as a consequence of recombination within a single gene. Accordingly we have isolated intergeneric intragenic recombinants in which different segments of the structural gene for the α chain of tryptophan synthetase [tryptophan synthase; L-serine hydro-lyase (adding indoleglycerol-phosphate), EC 4.2.1.20] are contributed from *Escherichia coli* and from *Salmonella typhimurium*. These organisms are appropriate for this study because the amino acid sequences of their α chains have been determined and shown to differ at 43 of their 268 residue positions (17). In addition, recombination within the *trp* operons and tryptophan synthetase α structural genes of these organisms has been observed (ref. 18; T. Mojica-a and R. Middleton, personal communication).

Abbreviation: Trp^+, tryptophan-independent.

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MATERIALS AND METHODS

Bacterial Strains Employed. (*E. coli* nomenclature for *trp* genes is used throughout.) The merodiploid *S. typhimurium cysB trpA109*/F'123 *cysB*$^+$ Δ[*tonB trpA229*] was constructed by transferring F'123 *cysB*$^+$ *trpE*$^+$*D*$^+$*C*$^+$*B*$^+$ Δ[*tonB trpA229*] from *E. coli* K-12 *his cysB*/F'123 *cysB*$^+$ *trpE*$^+$*D*$^+$*C*$^+$*B*$^+$ Δ[*tonB trpA229*] into *S. typhimurium cysB trpA109*. Cell mixtures were plated on indole agar containing citrate (which *E. coli* cannot use) as sole carbon source, thereby selecting for the transfer of *cysB*$^+$ to *S. typhimurium*. The resulting merodiploid was then used as the source of spontaneously arising tryptophan-independent (Trp^+) intragenic recombinants. The *trpA109* mutation of *S. typhimurium* was reported to be a small deletion at the operator-proximal end of *trpA* (19). We have found that it reverts to CRM^+ (production of crossreacting material) and to Trp^+ at a very low frequency (A. Schweingruber and C. Yanofsky, unpublished). We have purified the tryptophan synthetase α chain of one prototrophic revertant of *trpA109* and compared it with the α chain of wild-type *S. typhimurium* by peptide mapping of tryptic and chymotryptic digests. No differences were discernible. Δ[*tonB trpA229*] is a deletion in *E. coli* that removes *tonB* and the region of *trpA* specifying the last 20 or so amino acid residues of the tryptophan synthetase α chain (20). Strains with this deletion have never been observed to revert to prototrophy.

Selection of Intragenic Recombinants. A single colony isolate of the merodiploid *S. typhimurium cysB trpA109*/*E. coli* F'123 Δ[*tonB trpA229*] was suspended in saline and approximately 10^3 cells were inoculated into a series of tubes, each containing 5 ml of L broth. The cultures were grown overnight and the cells were concentrated, washed, and plated on minimal agar. Colonies appeared at a frequency of approximately 1 per 10^8 cells plated. Trp^+ colonies were picked and patched to a master plate of minimal agar and replicated to streptomycin/casein-hydrolysate agar spread with cells of *S. typhimurium leu500* Δ[*cysB supX trpEA*] *str*^{r}. Approximately 1–5% of the patched colonies transferred a *cys*$^+$ *trp*$^+$ plasmid to the streptomycin-resistant recipient. Several cysteine-independent, Trp^+, streptomycin-resistant colonies arising from transfers from different tubes were picked and purified. The Trp^+ plasmids were transferred from the *S. typhimurium* background into *E. coli* W3110 *his trp*Δ*EA2*, a strain lacking the

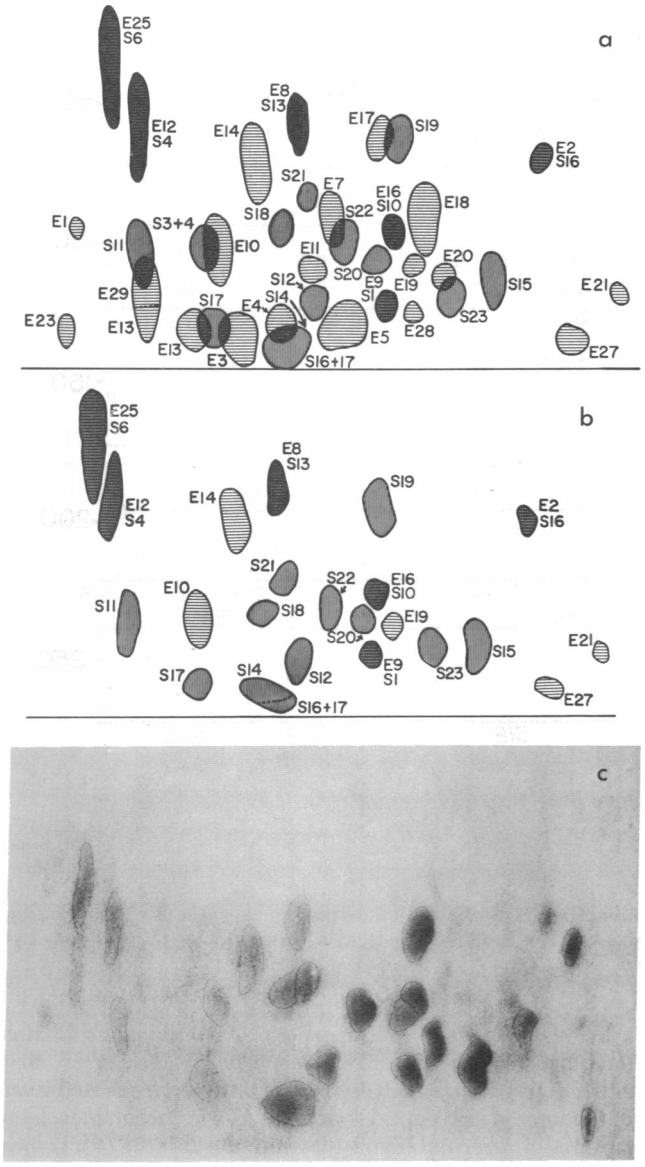

FIG. 1. Comparison of fingerprints of tryptic digests of the α chains of *E. coli* K-12, *S. typhimurium*, and hybrid strain E-S L23. (a) The major *E. coli* (prefix E, horizontal stripes) and *S. typhimurium* (prefix S, vertical stripes) tryptic peptides as they normally appear on fingerprints. (b) The major peptides identified on fingerprints of the hybrid proteins, with the source identified by the prefix E or S and horizontal or vertical stripes. (c) Photograph of a ninhydrin-stained fingerprint of a tryptic digest of the α chain of strain E-S L23. In general peptides were stained with fluorescamine because of the greater sensitivity of this reagent. Peptides not detected on the fingerprints are E6, E15, E26, E22, S2, S5, S7, S8, S9, and S24. Descending chromatography was run first (bottom to top, as shown) followed by electrophoresis from left to right at pH 3.5 (23).

entire *trp* operon, selecting for Trp^+ on Tris-minimal agar containing lactose as sole carbon source and supplemented with histidine. (*S. typhimurium* cannot use lactose as carbon source.) The resulting histidine-requiring Trp^+ strains were purified by streaking.

Protein Procedures, Enzyme Assays, and Antibody Inhibition Tests. The α chains of the three presumed *trpA* recombinants were purified by the standard procedure (21). These proteins were oxidized with performic acid (22) and digested with trypsin, chymotrypsin, or both enzymes (23, 24). Peptide maps were prepared as described previously (23, 24).

Table 1. Composition of relevant chymotryptic peptides

Source	Residues
	51–54
E. coli α	Gly 0.88, *Ile* 1.07, Pro 1.13, Phe 0.93
S. typhimurium α	Gly 0.91, *Val* 1.07, Pro 1.05, Phe 0.97
E-S α	Gly 0.95, *Ile* 1.02, Pro 1.03, Phe 1.00
	66–69
E. coli α	Asp 0.83, Ala 1.19, Thr 1.07, Leu 0.91
E-S α	Asp 0.87, Ala 1.07, Thr 0.99, Leu 1.07

Amino acid analyses, peptide procedures, and protein sequence analyses were performed as described (17).

α-Chain activity was determined in the indole + L-serine → L-tryptophan and the 3-indolylglycerol phosphate + L-serine → L-tryptophan reactions as described elsewhere (21). Partially purified *E. coli* tryptophan synthetase β_2 (25) was employed in assays of α chain activity. Anthranilate synthetase activity was assayed as described (21).

Antibody inhibition tests were performed with antisera to highly purified tryptophan synthetase α of *E. coli* and *S. typhimurium*. The antisera were generously provided by Stanley Mills. Three units of α chain (indole → tryptophan reaction) were incubated at 4° with sufficient antisera to inhibit 2 units of the α chain of *E. coli* W3110 or *S. typhimurium*. After 15 min the substrate mixture was added and the mixture was incubated for the usual assay period, 20 min, at 37°.

RESULTS

Isolation of Intragenic Recombinants with Hybrid α Chains. When suspensions of the hybrid merodiploid *S. typhimurium cysB trpA109/E. coli* F'123 *cysB*$^+$ Δ[*tonB trpA229*] are plated on selective agar, prototrophs appear at a low frequency (see *Materials and Methods*). A fraction of the prototrophs transfer F plasmids that carry a functional *trpA*. Each functional *trpA* presumably arose by genetic exchange in the unaltered segments of *trpA* of the parents. Three separately isolated Trp^+ strains, each bearing a recombinant plasmid over a chromosomal deletion of the entire *trp* operon, *trp*Δ*EA2*, were prepared for further study. The strains are designated E-S L23, E-S C85, and E-S N87.

Structure of Recombinant α Chains. Tryptic digests of oxidized α chains of the presumed *trpA* recombinants were examined by two-dimensional peptide mapping (Fig. 1). The tryptic peptide patterns obtained with the recombinant proteins were indistinguishable among themselves but differed from those of each of the parental proteins. In particular, peptides were recognizable from the amino terminal segment of the *E. coli* α chain (E10, E14, E19, E21, E27) and the carboxy terminal segment of the *S. typhimurium* α chain (S11, S12, S14, S15, S16 + 17, S18, S19, S20, S21, S22, S23) (see Figs. 1 and 2). *E. coli* tryptic peptide E23 (residues 100–120) was missing and all identifiable peptides beyond position 120 were of *Salmonella* origin.

Unfortunately peptides from the region of residues 36–109 are not readily recognized on tryptic peptide maps. Peptide maps of chymotryptic digests were therefore examined in an effort to localize the positions of sequence exchanges.

Peptide patterns of chymotryptic digests of the hybrid α chains were indistinguishable from each other but differed from those of the α chains of the respective parents. All peptide differences were explored by eluting the relevant peptides and analyzing their composition. Two peptides of interest were characterized (Table 1). Chymotryptic peptides representing

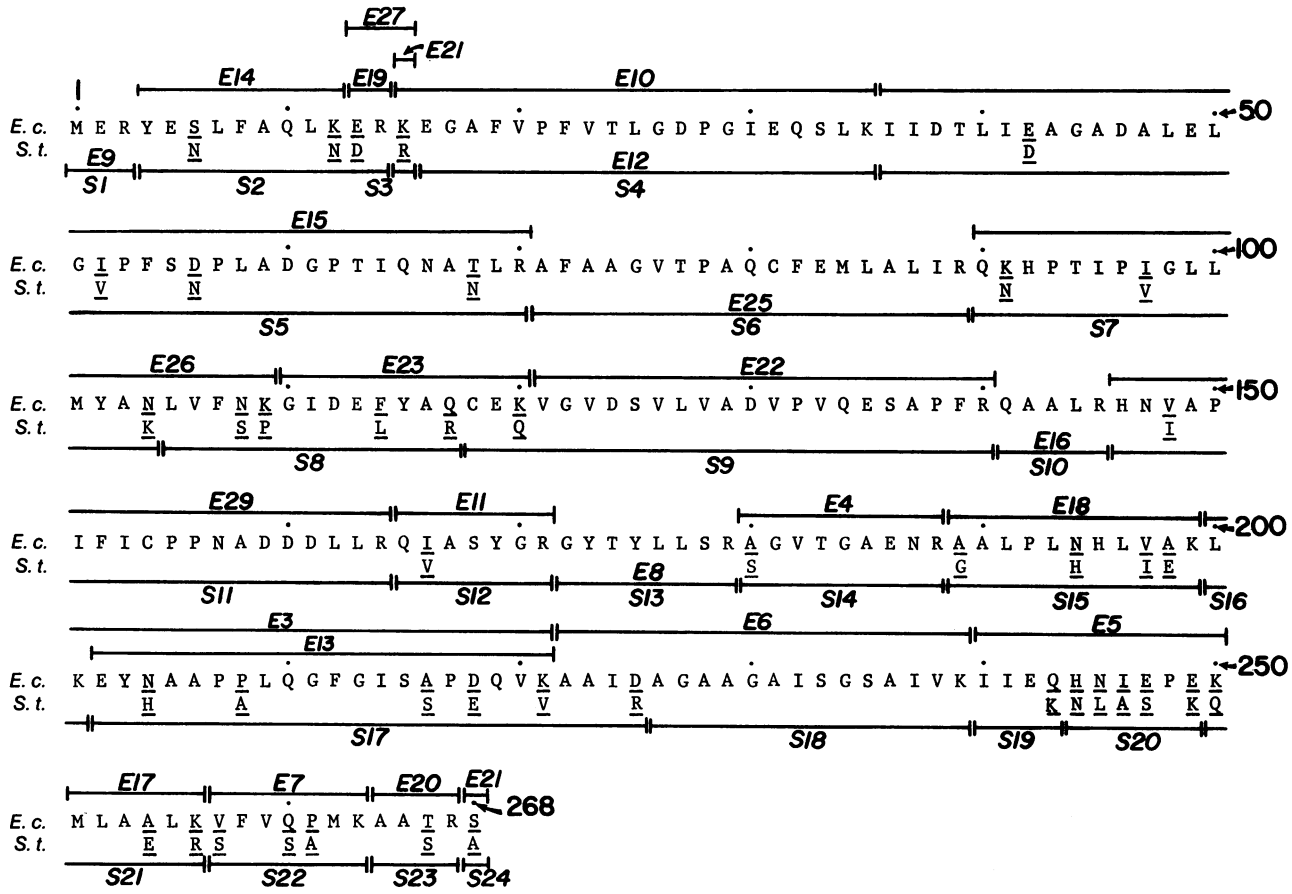

FIG. 2. Amino acid sequences of the α chains of *E. coli* (*E. c.*) and *S. typhimurium* (*S. t.*). Abbreviations used (1): A = Ala, C = Cys, D = Asp, E = Glu, F = Phe, G = Gly, H = His, I = Ile, K = Lys, L = Leu, M = Met, N = Asn, P = Pro, Q = Gln, R = Arg, S = Ser, T = Thr, V = Val, Y = Tyr. *E. coli* (prefix E) and *S. typhimurium* (prefix S) peptides are indicated.

residues 51–54 were at slightly different positions on fingerprints of *E. coli* and *S. typhimurium* digests. The corresponding peptide from the hybrid proteins was at the *E. coli* position and had the *E. coli* composition Gly,*Ile*,Pro,Phe rather than the *S. typhimurium* sequence Gly-*Val*-Pro-Phe. Similarly, the chymotryptic peptide containing residues 66–69 was at the same position on fingerprints of the *E. coli* digest and digests of the hybrid proteins, but there was no peptide at this position on fingerprints of *S. typhimurium* digests. As shown in Table 1 the *E. coli* and hybrid peptides have the same composition, Asp,Ala,*Thr*,Leu (Asn is converted to Asp during acid hydrolysis). The corresponding peptide of *Salmonella* should have the sequence Asn-Ala-*Asn*-Leu (Fig. 2). We believe we have located this peptide on fingerprints but we cannot be certain because it was contaminated by another peptide. These analyses of tryptic and chymotryptic peptides, plus the additional finding from automatic Edman degradation studies that residue 42 is Glu, establish that the hybrid α chains are of *E. coli* origin up to at least residue 69.

The relative net charges of the hybrid α chains were compared with those of the parental proteins on urea/acrylamide gels at pH 8.7. All hybrid chains migrated slightly more slowly than the parental proteins (which have the same charge) suggesting that they have one more positive charge. To explain this charge difference on the basis of the amino acid sequences of the parental proteins (Fig. 2) the switch from *E. coli* sequence to *S. typhimurium* sequence in the hybrids must have occurred either between residues 91 and 103 or between residues 110 and 116.

***In Vivo* and *In Vitro* Properties of Recombinant α Chains.** If a functional recombinant α chain were less active *in vivo* than either of its parental α chains we should observe elevated levels of *trp* operon polypeptides (relative to the parental levels) in cultures of α recombinants grown in the absence of a tryptophan supplement. This would occur because under such conditions the cell would be forced to derepress synthesis of its *trp* operon polypeptides so that tryptophan production would not become growth limiting. For example, *E. coli* strains with altered functional α chains with very similar structures, e.g., α chains that differ from their parental α chains by having Ser or Thr instead of Gly at position 211, produce elevated levels of the *trp* operon polypeptides yet do not have an altered cell growth rate (26). This test of enzyme function is particularly meaningful since it assesses *in vivo* α chain activity and stability.

The recombinant strains (*E. coli* genetic background) and *E. coli* strain W3110 were grown in parallel on three carbon-energy sources. There was about a 2-fold range in generation times on the different carbon sources but the growth rates of the recombinant strains were essentially indistinguishable from those of strain W3110 (Table 2). Extracts were prepared from each of the cultures and assayed for anthranilate synthetase and tryptophan synthetase α and β_2 activities. (Anthranilate synthetase is a complex composed of the polypeptide products of *trpE* and *trpD*.) Inspection of the measured enzyme levels (Table 2) reveals that, in comparison with *E. coli* W3110, the recombinants contain the same amount or very slightly more of the *trp* operon polypeptides. This suggests that the recom-

Table 2. Enzyme levels in exponential phase cultures

Strain	Generation time, min	Carbon-energy source	Specific activity: Anthranilate synthetase	Specific activity: Tryptophan synthetase β_2	Specific activity: Tryptophan synthetase α
E. coli W3110	78	Glucose	0.38	2.3	2.6
E-S L23	80	Glucose	0.47	2.8	2.6
E-S C85	84	Glucose	0.45	2.1	3.1
E-S N87	78	Glucose	0.47	2.7	3.4
E. coli W3110	101	Glycerol	0.27	1.6	1.9
E-S L23	101	Glycerol	0.31	2.1	2.4
E-S C85	88	Glycerol	0.35	2.4	2.9
E-S N87	86	Glycerol	0.34	2.5	3.2
E. coli W3110	48	Glucose/ACH	1.0	5.3	4.1
E-S L23	49	Glucose/ACH	1.1	6.2	6.3
E-S C85	47	Glucose/ACH	1.1	5.7	5.5
E-S N 87	44	Glucose/ACH	1.1	7.6	6.3

E. coli strain W3110 and strains E-S L23, C85, and N87 were grown in 200 ml of minimal medium supplemented with 0.2% glucose, 0.2% glycerol, or 0.2% glucose + 0.3% acid-hydrolyzed casein (ACH), each plus 30 μg of histidine per ml, to a cell density of 5×10^8 cells per ml. The cultures were chilled and the cells were collected by centrifugation and extracts were prepared by sonic disruption and assayed for the enzyme activities indicated. Specific activities are calculated per mg of protein (21).

binant α chains are approximately as active *in vivo* as the parental α chain under the test conditions imposed. Another indication that the hybrid α chains are highly active is the observation that 3-indolylglycerol phosphate is not accumulated by the recombinant strains. This intermediate always appears in culture filtrates of strains that have partially active α chains (27). Based on prior studies (26) we would expect that the *trp* enzyme levels of the recombinants would have been at least four times higher if they were growth-limiting. These conclusions should be qualified by the reservation that we have not examined the functional capacity of the hybrid α chains under a wide variety of environmental conditions.

The properties of the recombinant α chains were also examined in *in vitro* tests. Relative enzymatic activities in the physiologically significant reaction, 3-indolylglycerol phosphate + L-serine → L-tryptophan, and the α-stimulated β_2 reaction, indole + L-serine → L-tryptophan, failed to reveal a significant defect in the recombinant α chains (Table 3). The hybrid α chains also reacted indistinguishably from the parental chains when incubated with neutralizing antibodies prepared against purified α chains from the respective parents (Table 3). The thermostability of the hybrid α chains was compared with that of the parental strains (Fig. 3) and was intermediate under the test conditions employed (only E-S N87 data are shown; other recombinant proteins behaved identically).

Table 3. Enzymatic and immunological properties of hybrid polypeptides

Source of synthetase α chain	Relative enzymatic activity InGP → Trp/In → Trp	% Inhibition by antibodies to α chain of: *E. coli*	% Inhibition by antibodies to α chain of: *S. typhimurium*
E. coli	0.49	67	72
S. typhimurium	0.51	60	77
E-S L23	0.54	—	—
E-S C85	0.58	65	91
E-S N87	0.57	58	81

Analyses were performed on α polypeptide preparations of 50% or greater purity. Enzyme activity measurements and antibody inhibition tests were performed as described in *Materials and Methods*. InGP, 3-indolylglycerol phosphate; In, indole; Trp, tryptophan.

Natural Variation: The α Chain Specified by the ColVB *trp* Plasmid. In the course of related studies peptide maps of tryptic digests of the α chain specified by the ColVB *trp*$^+$ plasmid of wild-type *E. coli* strain K260 (28) were compared with those of *E. coli* strain K-12. One difference was noticed, displacement of the peptide containing residues 257–263. Analyses of this peptide revealed that Val replaced Ala at position 257. The properties of the α chain specified by this plasmid are indistinguishable from those of the α chain of *E. coli* W3110.

DISCUSSION

We have isolated and examined intergeneric intragenic recombinants from crosses of *E. coli* with *S. typhimurium* in an

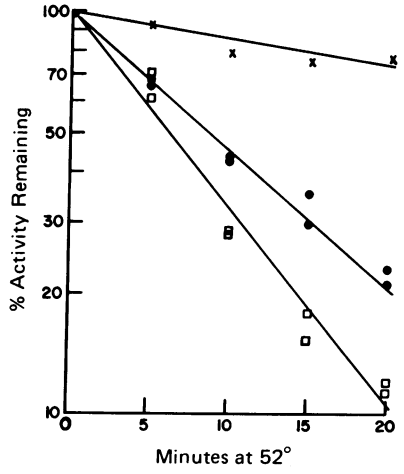

FIG. 3. Thermal stability of hybrid α chains. Two hundred units of each purified α chain were in 1 ml containing 0.05 M K phosphate buffer, pH 7.8, 1 mM EDTA, 1 mM dithiothreitol, 5% (vol/vol) glycerol, and 0.5% bovine serum albumin. The mixture was heated at 52° and samples were removed at the times indicated and added to cold 0.1 M Tris·HCl buffer, pH 7.8. Samples were assayed in the indole → tryptophan reaction in the presence of an excess of *E. coli* tryptophan synthetase β_2. ×, *E. coli* α; ●, E-S N87 α; □, *S. typhimurium* α.

effort to gain some understanding of the evolutionary basis of protein structure variation. The recombinants obtained were required to form functional hybrid tryptophan synthetase α chains. Recombinants presumed to be of this type have been isolated previously (T. Mojica-a and R. Middleton, personal communication). The three recombinants studied produce hybrid α chains which have similar primary structures. We assume that these proteins are specified by hybrid genes of near-identical sequences. The hybrid polypeptides have the *E. coli* sequence throughout their amino terminal 68 residues and the *S. typhimurium* sequence for the last 148 residues; thus they have at least 27 and 8 residue differences, respectively, from the α chains of *E. coli* and *S. typhimurium*. We do not know why the three hybrid α chains may be identical. Conceivably the parental mutants employed and the selective conditions imposed determined which recombinant classes would be recovered. Alternatively, genetic exchanges may have occurred preferentially in the regions of greatest nucleotide sequence homology in the corresponding α-chain structural genes.

In vivo and *in vitro* analyses were performed to evaluate the functional capacity of the hybrid α chains. They were found to be essentially equivalent to the α chains of their parents under the experimental conditions employed. This suggests that at least some of the amino acid sequence differences that exist in the α chains of *E. coli* and *S. typhimurium* may be noncritical to the function or survival of the respective proteins. If all the primary sequence differences were important or essential in their respective backgrounds, we probably would not have obtained hybrid proteins with near-normal activity.

Analyses of *in vivo* function have been performed with hybrid merodiploids which have *trpA* of *E. coli* and *trpB* of *S. typhimurium* and vice versa (29, 30). Such strains appear to be fully competent in tryptophan-synthesizing ability (30), despite the fact that their tryptophan synthetases are composed of subunits from different organisms. Other investigators have shown that the α chains of the two organisms interact more-or-less equivalently with the tryptophan synthetase β_2 subunit of *E. coli* (31).

Naturally occurring hybrid proteins have been recognized (1). The Lepore and anti-Lepore human hemoglobins, for example, have hybrid sequences and therefore undoubtedly represent the products of hybrid genes produced by genetic exchange between the genes for the β and δ chains of hemoglobin (32). In some cases such hybrid proteins have been shown to be abnormal (33).

We would like to extend our studies of intragenic recombination in *trpA* of *E. coli* and *S. typhimurium* by isolating recombinants with many structurally different hybrid α chains. With these we should be able to ascertain whether any hybrid α chains are catalytically defective and, if so, what fraction are of this type.

The authors are indebted to Drs. Elias Balbinder and Ronald Bauerle for providing stocks of *S. typhimurium* and to Drs. T. Mojica-a, R. Middleton, and G. Drapeau for informing us that functional intergeneric intragenic recombinants could be produced. We also thank Drs. Marcus Feldman and Naomi Franklin for their criticism of the manuscript. These studies were supported by grants from the U.S Public Health Service (GM09738) and the National Science Foundation (PCM73-06774 A03). C.Y. is a Career Investigator of the American Heart Association.

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