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Complementation In Vitro Between Mutationally Altered β_2 subunits of *Escherichia coli* Tryptophan Synthetase

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Cross-reacting β_2 subunits (CRMs) were purified from eight *trpB* missense mutants to test for complementation in vitro after urea dissociation and reaggregation. One CRM (B290, demonstrating "repairability," i.e., the appearance of enzymatic activity on combination with α subunits) was clearly positive with four others, all "non-repairable" CRMs resulting from mutations at three different but neighboring sites. One complementing pair, B290-B248, was studied in more detail and found, upon mixing purified proteins, to give complementation in the absence of denaturants. Complementation activity was low in each case. To study the mechanism of the modest increases in activity, we used a reduced β_2 subunit as an artificial CRM to form hybrids where both the amount of activity due to complementation and the amount of hybrid could be measured. (In a reduced β_2 subunit, the two pyridoxal phosphate cofactors have been chemically reduced by sodium borohydride and are covalently attached to lysine residues. This abolishes activity in the tryptophan synthetic reaction and causes the protein to migrate much faster than normal in acrylamide gel electrophoresis.) Reduced β_2 subunit formed hybrid dimers with the non-repairable CRMs B244 and B248 at pH 6.0, but no enzymatic activity appeared. On the other hand, when reduced β_2 subunit was mixed with B290 CRM at pH 6.0 to 6.6, an activity increase was seen that was proportional to the amount of hybrid. We conclude that hybrid formation is essential for complementation and that the mechanism of complementation in this system is the correction of a repairable active site on the B290 β chain by a conformational change occuring when hybrid dimer is formed. This type of complementation must be restricted to a small class of CRMs having a conformationally deformed active site. From the amount of hybrid present and the increase in activity, a specific activity of 50 U/mg was calculated for the hybrid containing reduced and B290 β chains. This value is slightly less than but close to the activity of the hybrid formed between reduced and normal β chains, shown earlier to have half the specific activity of the normal dimer.

Intragenic complementation was first observed in genetic experiments when diploid individuals bearing two different mutant versions of the same gene showed a wild or partially wild phenotype (13). This phenomenon has been studied in vitro with *Neurospora crassa* adenyl succinase (30) and glutamic dehydrogenase (2), *Salmonella typhimurium* imidazolglycerolphosphate dehydrase (20), and *Escherichia coli* alkaline phosphatase (24) and β-galactosidase (23), among others. In each of these cases, it is believed that the primary gene product aggregates into a multimer to become enzymatically active, and that the phenomenon of complementation results from formation of hybrid molecules containing subunits of both mutant types.

E. coli tryptophan synthetase (EC 4.2.1.20, L-serine hydrolyase [adding indole], and EC 4.1.2.8, indole-3-glycerolphosphate D-glyceraldehyde-3-phosphate-lyase) is a multimeric enzyme composed of four polypeptide chains ($\alpha_2\beta_2$). The multimer dissociates into A (α subunit) and B (β_2 subunit) components (7, 8, 29). There are no interchain covalent linkages in the native enzyme. Both the $\alpha_2\beta_2$ complex and the β_2 subunit can catalyze the formation of tryptophan from indole and serine (reaction 1).

$$\text{indole} + \text{L-serine} \rightarrow \text{L-tryptophan} \quad (1)$$

With ordinary potassium phosphate buffers, the β_2 subunit has 10% of the activity of the $\alpha_2\beta_2$ complex in this reaction, although under other conditions over 60% of the activity of the

complex can be seen (14). The β_2 subunit also causes a dramatic increase in the reaction weakly catalyzed by dissociated α subunit (reaction 2).

indole-3-glycerolphosphate $\rightleftharpoons$

indole + D-glyceraldehyde-3-phosphate (2)

This stimulation is also evoked by many mutant β_2 subunits (B-CRMs) lacking reaction 1 activity.

The β_2 dimer dissociates into free monomers in urea-containing buffers and can be reaggregated into native dimers by the removal of urea (16). It also dissociates spontaneously into monomers to a certain extent (15, 16). The β_2 dimer binds two molecules of pyridoxal-5'-phosphate (PLP); these two cofactor molecules appear to reside in identical active sites which can act independently (22). We have already shown that "half-reduced" enzyme, where one active site has been inactivated chemically, has one-half the activity of the native β_2 dimer (16).

Although free α-chains of *E. coli* tryptophan synthetase do not dimerize naturally, Jackson and Yanofsky (19) were able to form dimers artificially after partial unfolding in urea and then removal of the denaturant. They were able to recover activity from dimers constructed by annealing two different mutationally altered α chains. We present here the results of some complementation experiments done with mutant β_2 subunits in vitro. We will show that hybrid dimer formation is necessary for complementation and that the hybrid must have at least one mutant β chain belonging to the class earlier termed "repairable" (4).

MATERIALS AND METHODS

Mutants and their enzymatic character. The method of isolation and characterization of the mutants used in this study has been described (4, 6). Their relative position in a recombinational map of the *trpB* gene has also been determined (5). A recombinational map of the mutants used in this study is shown in Fig. 1.

All the mutants except *trpB21*, *trpB62*, and *trpB742* were isolated originally as double mutants in the *trpA2* (ochre) background. Double mutants *trpA2trpB21* and *trpA2trpB62* were prepared by transduction as described elsewhere (5). Strain *trpB742* was derived from a strain also containing the missense mutation *trpA3*. Mutant β_2 subunits (B-CRMs) retain to various degrees the enzymatic properties of the wild-type β_2 dimer (4–6). The classification and enzymatic character of B-CRMs used in our experiments is summarized in Table 1. One class, termed "repairable" (4), although nearly devoid of activity in reaction 1 by itself, regains activity in the presence of α subunits. Ammonium ions often work synergistically with α subunits in this process. "Conventional" B-CRMs retain only the ability to stimulate the α subunit in reaction 2. "Inert" B-CRMs react with antibodies made against the native molecules but are unable to stimulate α subunits, probably because they cannot properly associate with them. The CRM produced in strain *trpB21* (hereafter called the B21 CRM, or B21 for short) is interesting and exceptional for it cannot stimulate α chains in reaction 2 but can catalyze reaction 1 in the presence of ammonium ions. This suggests that it cannot combine with α subunits but is not irreparably damaged in its own enzymatic activity.

Growth of mutant strains. It proved more difficult to derepress *trpB* mutants than other *trp* auxotrophs, but the following method gave extracts which we judge to be at least half maximally derepressed. Mutants growing in 1 to 2 ml of complete medium were inoculated into 500 ml of Vogel-Bonner minimal medium E (28) supplemented with 0.2% glucose, 0.05% acid-hydrolyzed casein, and 4 μg of L-tryptophan per ml. After shaking overnight at 37 C, the entire culture was added to 10 liters of the same medium in a 25-liter fermenter. Cells were cultured at 37 C under vigorous aeration until a turbidity indicating 4×10^8 cells per ml had been reached. This culture was then transferred to 100 liters of prewarmed Vogel-Bonner medium E containing 0.2% yeast extract (Difco) and 8 μg of L-tryptophan per ml on a 150-liter fermenter. Under these growth conditions, 8 μg of tryptophan per ml is not a repressing level for our *trpB* auxotrophs. Cells were cultured at 37 C under vigorous aeration until a turbidity indicating a density of 6×10^8 to 8×10^8 cells per ml was reached. Growth often slowed at this point but resumed on the addition of 0.2% glucose. Growth was allowed to proceed to the early stationary phase, about 2×10^9 to 3×10^9 cells per ml, whereupon the cells were harvested by centrifugation and washed once in 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochlo-

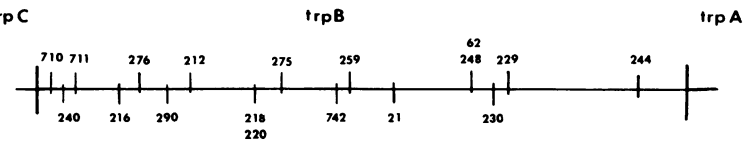

FIG. 1. *Recombinational map of the trpB gene. The relative positions of the markers used in this study are shown according to the best available information (5). The direction of transcription and translation is from left to right. Markers B62 and B248 are inseparable by recombination and produce similar CRMs. Markers B218 and B220 are inseparable by recombination but produce dissimilar CRMs. All mutations shown are revertible.*

TABLE 1. *Enzymatic properties of various trpB mutants*

TrpB mutation		Reaction 1 activity			Reaction 2 activity
Classification	Mutant site	No addition	With α subunit	With NH_4^+	
Repairable	B275 B290 B710 B711 B216 B218 B240	–[a]	+	+	+
Conventional	B62 B230 B244 B248 B212 B229	–	–	–	+
Inert	B220[b] B259 B276 B742[c]	–	–	–	–
Unusual	B21	–	–	+	–
Normal dimer		+	+	+	+
Reduced dimer		–	–	–	+

[a] Little or no activity, depending on the individual case.

[b] Although originally classified a conventional CRM, repeated assays on well-derepressed cell extracts demonstrate little or no reaction 2 activity. This protein also reacts poorly with antiserum in the radial immunodiffusion assay.

[c] Originally classified a conventional CRM, recent results indicate little or no reaction 2 activity.

ride buffer at pH 7.8. The yield was normally 250 to 300 g (wet weight).

Purification of CRMs. Slight modifications of the method for the normal β_2 subunit (29) were required. The primary modifications involved the temperature of the heat treatments, which were varied according to the heat stability of each mutant protein. The first heat treatment for CRMs B230, B248, and B62 was at the same temperature used for the wild-type protein. Only 53 to 54 C was used for the first heat treatment of the other five CRMs. The second heat treatment was adjusted to 79 to 80 C for B230 and B248, 77 to 78 C for B290, B244, and B62, 74 to 75 C for B21 and B710, 73 to 74 C for B275, and 72 to 73 C for B229. By this procedure all CRMs were obtained in 70 to 90% purity except for B21. When further purification was necessary to eliminate all extraneous bands on acrylamide gels, CRMs were filtered through a G-150 Sephadex column (2.5 cm diameter, 80 cm length) in 0.02 M potassium phosphate (pH 7.0) containing 10 mM β-mercaptoethanol and 10 μg of PLP per ml.

Assay of CRMs during purification. Repairable CRMs B290, B275, and B710 were assayed in reaction 1 in the presence of α subunits (25). B21 was assayed in the same reaction but with the addition of 3.5 M ammonium ions (added as the citrate) instead of α subunits. The remaining mutant proteins were assayed in reaction 2 in the presence of excess α subunits (25). B230, B244, and B62 were tested in the presence of 1 M salt-free hydroxylamine (10). Instead of hydroxylamine, PLP at 200 μg/ml was added for B248. B229 was assayed in the presence of 1.2 M ammonium ions and 1 M hydroxylamine.

Complementation after dissociation by urea. Dissociation and reaggregation were accomplished by the method used for normal and borohydride-reduced B protein (16). Partially purified CRM preparations were diluted to 2 mg of protein per ml, dialyzed separately against a 6 M urea solution for dissociation, combined in equal amounts in one dialysis bag, and reaggregated for 3 h as described for the wild-type enzyme (16). Controls included the unmixed component proteins. To determine whether complementation had taken place, the protein mixture was assayed in reaction 1 in the absence of α subunits by using 0.1 M potassium phosphate (pH 7.8) instead of the usual Tris buffer and sodium chloride (25).

Complementation without dissociative treatments. The two CRMs to be tested were dialyzed overnight against 0.1 M potassium phosphate buffer at the indicated pH with supplements of 1 mM β-mercaptoethanol and 10 μg of PLP per ml. The CRMs were then mixed together and kept at 0 C to minimize denaturation. Samples were removed at timed intervals and assayed by the same method as the dissociated and reaggregated samples.

Complementation by mixing two crude extracts. Extracts of each mutant were prepared by sonic distruption in 0.1 M potassium phosphate buffer (pH 7.8) containing 1 mM β-mercaptoethanol. Amounts of the two extracts providing equal amounts of the two CRMs were mixed together and allowed to stand for a few hours in an ice bath; the increase in activity was then measured in the presence of α subunits. The amount of CRM in crude extracts was measured by radial immunodiffusion (11).

Formation of hybrid between CRMs and borohydride-reduced β_2 dimer. Reduced β_2 dimer was prepared as described earlier (16). CRMs and reduced β_2 dimer were mixed together and dialyzed against 0.1 M potassium phosphate buffer containing 10 mM β-mercaptoethanol at various pH values. The buffer was changed at 2-h intervals for 6 h of dialysis. These samples were divided. One portion was immediately placed on analytical acrylamide gel columns without the addition of dithioerythritol, and the enzymatic activity of the other was assayed without addition of α subunit. Acrylamide gel electrophoresis was conducted with either the Tris-glycine (26) or Tris-asparagine (17) buffer systems.

Scanning gels to measure the relative amounts of proteins. After staining by amido schwartz, the gels were scanned at 550 nm in a Gilford spectropho-

tometer, and the percentage of CRM, hybrid, and reduced β_2 dimer was calculated.

Other methods. Protein was determined by the method of Lowry et al. (21) with bovine serum albumin as standard. Values obtained with the standard solution were corrected slightly to the dry weight of β_2 subunit (29). Samples of α subunit used in the assay of tryptophan synthetase were purified by the procedure of Henning et al. (18).

RESULTS

Complementation after urea treatment. Mixtures of all possible pairs of eight purified CRMs were dissociated in urea and allowed to reaggregate under conditions that allow restoration of the activity of the wild-type β_2 dimer (Table 2). One of the CRMs belonging to the repairable class, B290, is clearly positive with B248, B62, B229, and B230. The B290-B21 pair is probably also positive. The admixture of B21 with B275, B248, B229, and B230, as well as the B275-B62 combination, may give very weak complementation but cannot be evaluated.

Complementation without exposure to denaturants. There is evidence from ultracentrifugal experiments at low protein concentrations that the β_2 dimer is in equilibrium with a small amount of monomer (15, 16). If this is true, it seems reasonable that mixing two complementary CRMs together should result in the appearance of enzymatic activity due to hybrid dimer formation. The B290-B248 pair was selected for this experiment. The time-dependent increase of activity after mixing these CRMs in dilute phosphate buffer is shown in Fig. 2. The enzymatic activity shown by the B290 control did not increase with time. Under the conditions used, the amount of activity, and therefore probably the amount of hybrid dimer, continued to increase in the experimental tubes kept at 0 C for several hours. Activity levels reached at pH 8.0 were appreciably higher than those at pH 7.0. Further experiments showed that complementation without denaturants can take place within a considerable pH range around neutrality, but is best slightly on the alkaline side.

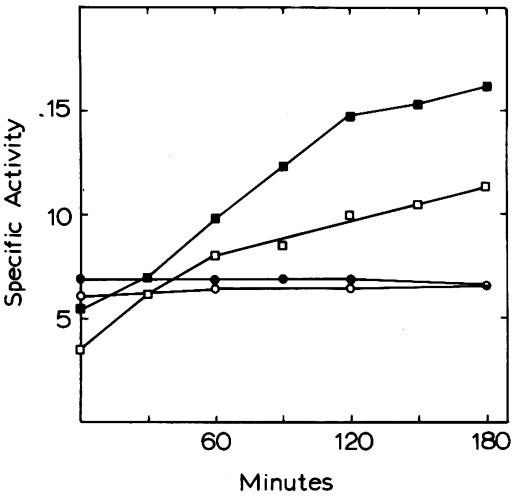

FIG. 2. *Complementation between B290 and B248 CRMs in the absence of denaturants. Results at pH 7.0 (open symbols) and pH 8.0 (closed symbols) are shown. The experimental mixtures, prepared as described in the text, had protein concentrations approximately equal to those in the control tubes with each CRM alone. The B248 controls were without activity and are not shown. Specific activity in the B290 controls (circles) and the experimental mixtures (squares) are presented as a function of time at 0 C.*

Optimal pH for complementation after urea treatment. The urea system was used to define the pH optimum for complementation to separate the known effect of pH on the dissociation constant (15) from its effect on the reaggre-

TABLE 2. *Complementation after dissociation with urea*

CRM	Complementation[a] (U/mg of protein)						
	B275	B21	B248	B62	B229	B230	B244
B290	0.48[a]	2.89	8.78	7.95	4.30	6.75	0.42
B275		1.11	−0.55	1.45	0.42	−0.65	−0.85
B21			1.89	0.47	2.07	1.17	−0.53
B248				−0.22	−0.12	−1.93	−2.33
B62					0.75	0.68	−0.12
B229						−0.44	0.30
B230							−2.37

[a] Specific activity of a mixture of the two CRMs less any activity shown by the unmixed controls subjected to the same operations. Control specific activities were below 4 U/mg for all CRMs except B275 (13 U/mg) and B21 (7 U/mg), and for conventional CRMs were usually around 1 U/mg. The complementation reaction was shown to be complete after the 3-h reaggregation time.

gation phenomenon. The regain of activity observed in the urea system showed a slight pH dependency (Table 3). The pH optimum in Tris buffer is 8.3. Potassium phosphate buffer appears to be clearly superior to Tris buffer for complementation, however.

Complementation by mixing crude extracts. Purified CRMs positive in urea-dissociated complementation experiments also gave positive results in mixing experiments without urea treatment. If it is reasoned that dissociation and reaggregation of β_2 dimers should not be greatly influenced by the presence of other proteins, simple mixing of crude extracts should suffice for the detection of positive complementation. Amounts of CRM in each extract were estimated by radial immunodiffusion to permit addition of equal amounts of two CRMs, and the complementation analysis was extended by this means to include 15 mutants (Table 4). The B290-B248 and B290-B230 pairs which were positive in urea-dissociated complementation were also positive in this experiment. One of the additional positive pairs found by the crude extract screening, B710–B244, was checked by mixing purified CRMs, and the result was confirmed. Therefore, with one exception which will be discussed, screening for complementation by mixing crude extracts seems valid.

TABLE 3. *Optimal pH for the B290-B248 complementation*

Reaggregation buffer[a]	Activity observed (U/mg)
Tris-hydrochloride, pH 8.0	4.54
Tris-hydrochloride, pH 8.3	5.80
Tris-hydrochloride, pH 8.6	5.14
Potassium phosphate, pH 8.0	7.01

[a] The reaggregation buffer was supplemented with 0.1 M β-mercaptoethanol and 30 μg of PLP per ml. Otherwise the usual urea-dissociation technique was used (see Materials and Methods).

There seem to be two kinds of repairable CRMs. Some, like B710 and B290, show positive complementation with four or five different β chains. Others, like B275, B216, and B711, have no positive pairs. The reasons for this difference are not clear. One possibility is that certain CRMs do not dissociate easily at neutral pH.

Three repairable CRMs, B240, B710 and B711, are located close to each other near the N-terminus of the protein, yet they differ strikingly in their complementation patterns. In certain cases, B290-B212 for example, the mutational sites for a positive pair are located very close to each other. From these results, there seems to be no obvious relationship between the complementation "map" and the recombinational map.

There were no clearly positive pairs among the combinations of non-repairable CRMs. When mixed, B276 and B244 gave an activity slightly above the level chosen as significant, but both the control activities and the activity of the mixture were low in this case, and complementation probably would not occur on

TABLE 4. *Complementation on mixing crude extracts*[a]

CRMs	Complementation													
	B230	B248	B259	B742	B275	B218	B220	B212	B290	B276	B216	B240	B710	B711
B244 (1)[b]	−	−	−	−	−	−	−	−	−	+	−	−	+	−
B230 (4)		−	−	−	−	−	−	−	+	−	−	−	+	−
B248 (5)			−	−	−	−	−	−	+	−	−	+	+	−
B259 (9)				−	−	−	−	−	+	−	−	−	+	−
B742 (9)					−	−	−	−	−	−	−	−	−	−
B275 (11)						−	−	−	−	−	−	−	−	−
B218 (12)							−	−	−	−	−	−	−	−
B220 (12)								−	−	−	−	−	−	−
B212 (17)									+	−	−	+	+	−
B290 (17)										−	−	−	−	−
B276 (18)											−	−	−	−
B216 (19)												−	−	−
B240 (21)													−	−
B710 (21)														−
B711 (21)														

[a] The test was considered positive if the mixture showed at least 30% more activity than the most active control when 100 U of α subunit were added per tube.

[b] The numbers in parentheses are the regions of the *trpB* gene where the mutational site is found. Regions are numbered sequentially from the *trpA* to the *trpC* end and were determined by a set of overlapping deletions (6).

mixing purified proteins. We believe the positive result in this instance to be an artifact of the method.

Hybridization of reduced β_2 dimer and CRMs in the absence of denaturants. One feature of the positive complementations we have studied is that the level of activity achieved is low, far less than that of wild-type enzyme. Another is that one member of the pair is always a repairable CRM. The ability of these CRMs to regain reaction 1 activity in the presence of α chains and/or ammonium ions (3; S. Kida, unpublished results) suggests that their active site is almost intact. They can be complemented by conventional CRMs showing no sign of having a functional active site for reaction 1. In assays, conventional CRMs resemble reduced β_2 subunits. Because of the negative charges on the cofactor, however, reduced β_2 subunits migrate much faster than normal β_2 dimers in acrylamide gel electrophoresis (16). (Only one of the eight purified CRMs differs in electrophoretic mobility from the normal enzyme, and it does not show positive complementation with any others.) Therefore, we used reduced β_2 dimer to form artificial hybrids in order to correlate the increase in activity with the amount of hybrid formed. We determined that reduced and normal β_2 dimers would spontaneously produce large amounts of hybrid at pH values around 6.5. Two typical, conventional CRMs, B244 and B248, were chosen as controls and mixed with reduced β_2 dimer at pH 6.0. Although large amounts of hybrid could be detected by electrophoresis (Fig. 3), no enzymatic activity appeared. Next, B290, a repairable CRM, was mixed with reduced β_2 dimer at several pH values between 7.8 and 6.0. The amount of hybrid dimer increased as the pH decreased (Fig. 4). Almost no hybrid was detected at pH 7.8, very slight amounts were found at pH 7.2 and 6.8, and larger amounts were seen at pH 6.4 and 6.0. An increase in activity was found only in the mixtures were hybrid dimer appeared (Fig. 5). Similar results were obtained with other repairable CRMs such as B275 and B710. From these experiments it is concluded that hybridization is essential for complementation to occur between the CRMs, but in addition one of the members of the pair must have a repairable active site.

Specific activity of hybrid dimer containing reduced β monomer and B290. It seemed important to estimate the specific activity of the hybrid dimer showing positive complementation and compare it to wild-type enzyme. Reduced β_2 dimer and B290 CRM were mixed

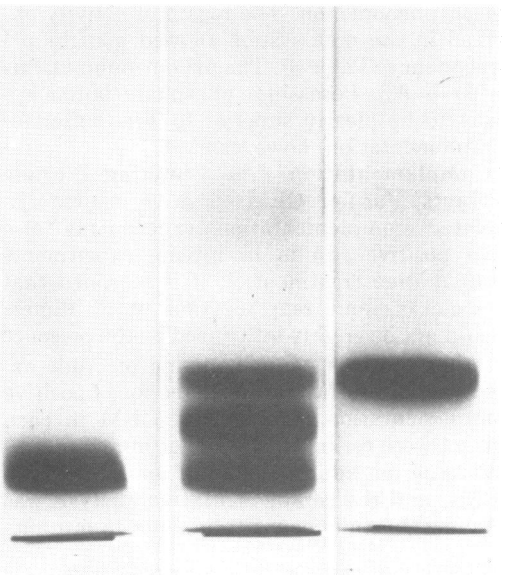

FIG. 3. *Formation of hybrid dimer on mixing reduced β_2 dimer and B244 CRM. The result of mixing these two proteins at pH 6.0 is shown. Left gel, Reduced β_2 dimer; middle gel, mixture of the two proteins; right gel, B244 CRM. The direction of migration was from top (cathode) to bottom in 6.0% acrylamide gel with the Tris-asparagine system.*

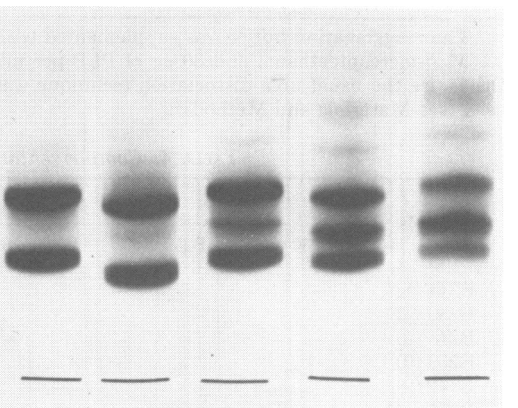

FIG. 4. *Formation of hybrid dimer on mixing reduced β_2 dimer and B290 CRM. Left to right, the mixtures were brought to pH 7.8, 7.2, 6.8, 6.4, and 6.0. The direction of migration was from top (cathode) to bottom in 7.5% acrylamide gel with the Tris-glycine system.*

together at pH 6.8, 6.6, and 6.4. Figure 5 presents the total activity in the mixture and controls (left bar), and the percentage of hybrid observed (right bar, diagonal hatching). The amount of hybrid corresponds approximately to the activity increase. Some decrease was observed in the control activity at the lower pH

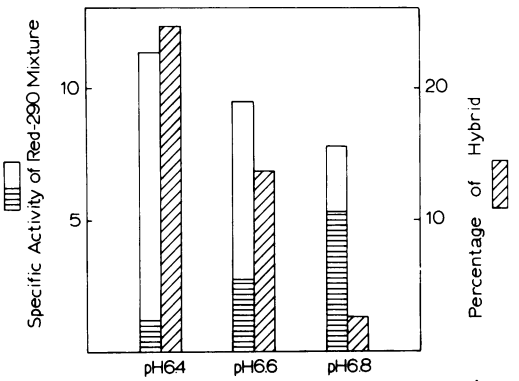

FIG. 5. *Relationship between the enzymatic activity and amounts of hybrid in mixtures of reduced β_2 dimer and B290 CRM held at various pH values. The left member of each pair of histograms shows the activity of the mixture (total height) and the B290 control (shaded part). The reduced protein control was without activity. The right member of each pair (diagonal hatching) shows the percentage of hybrid in each mixture.*

values because this activity comes primarily from B290 CRM, which is more fragile than reduced β_2 dimer below neutrality.

In calculating the specific activity of the hybrid dimer from this increase in activity and the percentage of hybrid, a value of 55 U/mg was obtained at pH 6.4 and a value of 44 U/mg was obtained at pH 6.6. It is already known that the specific activity of hybrid containing normal and reduced β chains is one-half that of the original β_2 dimer (15). The reduced dimer used in the present experiment was prepared from β_2 dimer having a specific activity of 120 U/mg in this assay. It would be predicted that the specific activity of a hybrid containing a reduced and a B290 β chain should be, as a consequence of conformational correction of the latter, similar to or slightly lower than that of the reduced, normal dimer. It therefore seems reasonable that the hybrid containing reduced and B290 chains has a specific activity around 50 U/mg.

DISCUSSION

Pairs of CRMs that are positive in complementation experiments involving urea treatment are usually positive also when complementation is sought after simply mixing crude extracts. In the latter case, hybrid dimers probably form as a consequence of a natural equilibrium between dimers and monomers (15, 16). In most instances, mutant β monomers can associate indiscriminately with other mutant β chains or with normal chains bearing the reduced form of the PLP cofactor, so an equilibrium exists between each homologous dimer, the heterologous dimer, and the two individual monomers.

Apparently the pH optimum for dissociation is not necessarily the best pH for complementation. We observed a complementation pH optimum at 8.0 to 8.3; perhaps this pH best favors both dissociation and reaggregation to produce the activity heterologous dimer. A different pH dependency is observed in hybridization between CRMs and reduced β_2 dimer. The amount of hybrid increases as the pH decreases between 7.8 and 6.0, notwithstanding the fact that reduced β_2 dimer shows no detectable dissociation in this pH range (15). This lower pH optimum seems peculiar to hybrids containing the reduced β monomer. Complementation between B290 and B248 in the absence of denaturants is no better at pH 6.4 than at 7.0 or 8.0, for example. Normal β_2 dimers and many mutant CRMs begin to precipitate at pH 6.4. When the reduced dimer has hybridized with B290 at pH 6.4, the mixture can be returned to pH 7.8 by dialysis, and the ratio of hybrid to homologous dimers is changed very little by the increase of pH. It appears that a pH of 6.0 to 6.4 is necessary for the formation of a considerable amount of hybrid containing reduced β chains, but is not essential for its maintenance. The reason for this phenomenon remains unknown.

In relating a complementation map to the recombinational map of the *trpB* gene, it might be assumed that there would be a spatial distribution of mutations affecting particular functions of the protein molecule, for example, the attachment sites for certain substrates. The polypeptide is folded into a globular molecule, however, and the location of specific functions need not be confined to one point on a linear polypeptide chain. Furthermore, proteins differing in their enzymatic character, such as repairable and conventional CRMs, may be obtained from recombinationally inseparable mutant sites (6). This fact suggests that the substitution of different amino acids at a particular site can have very different effects on the conformation of the protein molecule. In our case, where complementation occurs by conformational repair, it is difficult to perceive any relationship between the genetic map and the complementation map.

The amount of complementation activity is low in this system, in contrast to some others (23, 24). Fan et al. (12) note that the amount of complementation activity observed with alkaline phosphatase mutants is a function both of the amount of hybrid formed and of the activity

of the hybrid. In cases where we failed to observe complementation between two mutants, we are not certain that hybrid was in fact formed. When complementing a CRM with reduced β_2 subunit, however, the amount of hybrid is easily observable. Under the best conditions we have found, a maximum of 30% hybrid was formed between CRM B290 and the reduced β_2 dimer.

What is the mechanism of complementation when a heteromultimer composed of two different mutant polypeptide chains has more activity than a homomultimer? Crick and Orgel (9) proposed conformational correction as an explanation for this phenomenon, and we believe this applies to our case. This hypothesis is easily visualized if both polypeptides aggregate into a multimer containing a single, active site. Commonly the problem is more complicated than that, however. With *E. coli* β-galactosidase (27), any mutation near the C or N terminus will complement with mutant polypeptide chains (or fragments of chains) having these C- or N-terminal regions intact (α or ω complementation). Our results showing some mutants near the ends of the *trpB* gene unable to complement seem to rule out this sort of mechanism. Apte and Zipser (1) have reported the occurrence in *rec*$^-$ merodiploids of active polypeptide chains synthesized from two genes. All of our complementation experiments were performed in vitro with proteins that had been synthesized in haploid cells, ruling out the occurrence of this mechanism also. We have not attempted to show complementation in vivo. In our case, it appears that the active site of one chain (B290, for example) is correctable by hybridization with other chains (B248 or reduced β-chains) because the B290 active site is almost intact to begin with. In contrast, non-repairable CRMs such as B244 cannot themselves be corrected by hybridization with any β chain. If one repairable β chain were to form a hybrid with another repairable one, this hybrid might possibly have a specific activity almost equivalent to that of the wild type. We have not found such a pair, however. The data we have obtained are most compatible with a model in which each of the two active sites on the β_2 dimer is formed from the amino acids of one β chain, but the deleterious effect of certain mutational substitutions can be alleviated by conformational correction operating between the subunits of a dimeric structure.

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