The Formation and Properties of Dimers of the Tryptophan Synthetase α Subunit of Escherichia coli

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SUMMARY

The normally monomeric α subunit of Escherichia coli tryptophan synthetase forms dimers and higher order aggregates following exposure to high urea concentrations and removal of the urea by dialysis. The maximum yield of α chain dimers is approximately 2%. Dimers of certain combinations of different enzymatically inactive mutant α monomers exhibit the missing enzymatic activity. In such heterologous dimers, the reconstituted active site has at least 50% of the activity of the active site of the native wild type α monomer.

α chain dimers form an enzymatically active complex with the tryptophan synthetase β2 subunit. However, an α chain dimer can bind only a single β2 molecule. The α chain dimer dissociates into α monomers when heated at 51°. A tentative model of the structure of an α chain dimer is presented.

Studies with RNase A have shown that 2 differently inactivated molecules of this normally monomeric protein can intersect to reconstitute a functional active site region from unmodified portions of the different monomers (1–4). This case differs from other examples of enzyme complementation in vitro (5–7) in that partial denaturation and heterologous association of monomers are involved rather than exchange of subunits of a multimeric enzyme. In this paper, we describe an example of complementation in vitro analogous to that of RNase A; in this case complementation is observed between different mutationally altered forms of a normally monomeric protein. The protein studied is the α subunit (A protein) of the tryptophan synthetase of Escherichia coli.

Tryptophan synthetase is composed of two nonidentical and easily separated subunits, designated α and β2 (8, 9). The α subunit is a single polypeptide chain, 267 amino acid residues in length, whose primary structure is known (10). It is normally monomeric and does not form dimers or higher order multimers at high protein concentrations (11). The β2 subunit is composed of two identical polypeptide chains which do not dissociate detectably under physiological conditions (11, 12). The fully associated tryptophan synthetase complex is the tetramer α–β2–α (8). When the β2 subunit is in excess, an α–β2 complex exists, is stable, and has half of the enzymatic activity of the α–β2–α complex (8, 11). The tryptophan synthetase complex catalyzes the following major reactions (9):

\[
\text{Indole + L-serine} \rightarrow \text{pyridoxal-P} \rightarrow \text{L-tryptophan} \quad (1)
\]

\[
\text{InGP} \rightleftharpoons \text{indole + glyceraldehyde-3-P} \quad (2)
\]

\[
\text{InGP} + \text{L-serine} \rightarrow \text{pyridoxal-P} \rightarrow \text{L-tryptophan + glyceraldehyde-3-P} \quad (3)
\]

Reaction 3 is thought to be the physiologically important reaction and to be a distinct reaction, not simply the sum of the other two (9, 13). The α subunit alone has slight activity in Reaction 2 and the β2 subunit alone has slight activity in Reaction 1. Neither component by itself has activity in Reaction 3 (9). Mutational alterations are known which abolish the inherent activity of each subunit (14, 15).

Many mutant α subunits have been isolated and shown to have single amino acid differences from the wild type α subunit (16–20). Such mutant proteins have the following characteristics: they lack 99% or more of the wild type activity in Reactions 2 and 3 when they are in complex with the β2 subunit; they also lack the low level of Reaction 2 activity exhibited by the wild type α subunit; and they are fully active in the α–β2–α complex in catalyzing Reaction 1. These properties suggest that mutant α subunits should be ideal material with which to investigate complementation in vitro of a normally monomeric protein. Mutant subunits can be readily detected and determined quantitatively by virtue of their ability to catalyze Reaction 1 when in complex with β2. Complementation between differently altered α chains should manifest itself by the appearance of activity in one or both of the reactions in which the mutant α subunits are deficient. In this and the accompanying paper (21) we describe the properties of enzymatically active dimers formed from the wild type α subunit and from different mutant α subunits. This paper is concerned primarily with the formation and properties of the active dimer species. The following paper is devoted to an examination of complementation by fragments of the α sub-

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† The abbreviation used is: InGP, indole 3-glycerol phosphate.
unit and to a survey of which pairs of mutant α subunit comple-
ment in vitro.

EXPERIMENTAL PROCEDURE

Organisms Used—The organisms used in these studies are deriva-
tives of the E. coli K12 Ymel strain. The trpA mutants were
isolated by penicillin selection after ultraviolet mutagenesis. The
Strains trpA38/F′trpA38 and trpA46/F′trpA46 contain an F′dist trp
Col BV episome (22) and are diploid for the trpA gene. TrpA38
and trpA46 are missense mutants with alterations in the operator-
proximal and operator-distal regions of the trpA gene, respec-
tively (16).

The double mutant trpETSAlO9 carries the undistinct syn-
thetase missense mutation trpETS and the trpA gene ochre mu-
tation trpA109. TrpETSAlO9 was used as a source of β sub-
unit for InGP to indole assays in order to avoid problems with a
phosphatase present in extracts of strains which accumulate
InGP.

Growth of Bacterial Strains—TrpA mutants were grown in
100 liter batches in a New Brunswick Permacell in minimal me-
dium (23) supplemented with 1% glucose, 0.01% acid-hydrated
casein, 7.8 mM (NH4)SO4, 19 mM K3HPO4, and 0.03 mM indole.
TrpETSAlO9 was grown just to stationary phase in 16-liter batches
of minimal medium supplemented with 0.4% glucose, 0.05% acid-hy-
droyzed casein, and 0.021 mM indole in 20-liter carboys.

Purification of Proteins—The wild type and mutant α subunits
used in these experiments were purified by a modification (24)
of the method of Henning et al. (25). The α preparations used
generally had specific activities in the indole to tryptophan re-
action at least 50% that of the pure protein.

The wild type β subunit was partially purified from extracts of
trpETSAlO9 by the method of Wilson and Crawford (12), except
that 0.1 M KPO4, pH 7.0, was substituted in all cases for
0.1 M Tris, pH 7.8. Purification was carried through the first
ammonium sulfate fractionation.

Reagents—InGP and 14C-InGP were synthesized enzymati-
cally as described by Hardman and Yanofsky (26). 1-(o-
Carboxyphenylamino)-1-deoxyribulose 5-phosphate was syn-
thesized by the method of Smith and Yanofsky as modified by
DelMoss (25). Salt-free hydroxylamine was prepared from
hydroxylamine hydrochloride by the method of Davie (27) and
was stored at −15°. Urea and guanidine hydrochloride, both ultra
purer grade, were purchased from Mann. All other reagents
obtained commercially and used without further purifica-

tion.

Enzyme Assays—Procedures used for measuring the enzymatic
activities of the α and β subunits in each of the major reactions
catalyzed by the tryptophan synthetase complex have been de-
described (28). All assays were carried out in a final volume of 1.0
ml at 37°. Incubation was usually for 20 min. Where longer
incubation times were used, the linearity of the reaction with
time was verified. Indole to tryptophan activity was determined in
a mixture containing enzyme, 0.4 mM indole, 60 mM α-L-serine,
0.04 mM pyridoxal phosphate, 0.18 M NaCl, 0.1 M Tris-HCl (pH
7.8), and 15 to 20 units of α or β subunit depending on whether β or α
was being assayed. Disappearance of indole was followed.
InGP to indole activity was determined with the same mix-
ture, except that 0.1 to 0.4 mM InGP was substituted for the
indole. After incubation, the InGP was oxidized to indole-3-
aldehyde with an excess of metaperiodate at pH 5.0. The in-
dole 3 aldehyde was extracted into ethyl acetate and absorbance
was determined at 290 nm (29). The difference in A290 between
the experimental sample and a control sample lacking enzyme
was used to calculate the amount of InGP converted to trypt-
ophan. A290 of 11.4 is equivalent to 1.0 mm InGP under these
conditions (30). Indole to tryptophan activity was routinely measured
in a mixture containing enzyme plus 0.1 mm 14C-InGP, 2.10e
epm pm mole, 0.08 mM pyridoxal-P, 0.6 M salt-free hydroxyl-
amine, 0.05 mM KPO4 (pH 7.0), and sufficient α or β subunit to
saturate the component being assayed. Hydroxylamine, 0.6 M,
has been found to give a 30- to 40-fold stimulation in the rate of
the InGP to indole reaction (31). After incubation, the reaction
was stopped with 0.2 ml of 1.0 N NaOH. Indole was then ex-
tracted with 2.0 ml of toluene, the mixture was centrifuged
briefly, and 1.0 ml of the toluene layer was counted in a Packard
liquid scintillation spectrometer.

InGP synthetase activity was measured in a mixture contain-
ing enzyme plus 0.2 to 0.4 mm (1-(o-carboxyphenylamino)-1-
deoxyribulose 5-phosphate and 0.1 M Tris-HCl buffer, pH 7.8
(28). InGP content was measured as described above.

One unit of activity in any of the reactions is defined as that
activity which causes the disappearance of 0.1 μmole of sub-
state or the appearance of 0.1 μmole of product in 20 min at 37°.
One unit of each tryptophan synthetase subunit is that amount of
protein which gives 1 unit of activity in the presence of an
excess of the other subunit. Unless otherwise stated, all refer-
ences to "units" in this paper refer to units of activity in the
indole to tryptophan reaction. For convenience in evaluating
data, we have adopted the following unit as a measure of the
extent of complementation. One "complementation unit" is
equivalent to a value of 1·10−4 for the ratio

Units of InGP to indole activity
Units of indole to tryptophan activity

This expression was used because it corrects for losses of indole
to tryptophan activity associated with the conditions required
for complementation. The term "complementation" is used in
this paper to denote instances in which there is an increase in
the above ratio (complementation units) when a mixture of mu-
ant α subunits is suitably treated.

The heat stability of enzyme preparations was determined in
the presence of 0.6% bovine serum albumin, 0.1 M KPO4 (pH
7.0), and 1 mm dithiothreitol at 51° (32). Protein was measured
by the procedure of Lowry et al. (33).

Sucrose Gradient Centrifugation—Sucrose gradients were 5 to
20% (w/w) in 0.1 M KPO4 (pH 7.0). When additional compounds
were present in the gradients, the sucrose solutions were made up
immediately prior to use. The gradients had a volume of 4.8 ml.
Sample, 0.2 ml, was layered on top. After running at 4° in a
Spinco model ZU or model L2-65B at 50,000 rpm in a SW50L ro-
tor, the rotor was allowed to coast to a stop. Fifty-three 5-drop
fractions were generally collected.

Sephadex Gel Filtration—Depending on the volume of protein
solution to be chromatographed, a column either 1 x 100 cm or
a 2 x 60 cm of Sephadex G-100 was used. In some experiments,
Sephadex G-100 superfine was used. All experiments were per-
formed at 4°. In most cases, fractions of constant volume were
collected and collected after application of the sample to the
column. Blue dextran 2000 (Amax) was used to determine the
void volume.
Dimers of Tryptophan Synthetase α Subunit

Fig. 1. Chromatography of urea-treated and control preparations of wild type α subunit. A, 10 mg of highly purified (maximum specific activity) α subunits were treated with urea, dialyzed, and chromatographed on a column of Sephadex G-100, 2 × 60 cm. The elution buffer was 0.1 M KPO₄ (pH 7.0), and the flow rate was approximately 3 ml per hour. The protein concentration in the urea solution was 5 mg per ml. B, chromatography of 20 mg of untreated α subunit. Procedure and column were the same as in A. Absorbance at 280 nm, •, indole to tryptophan activity, O; indole to tryptophan activity on expanded scale, ▲.

Protocol for Dimer Formation and Assay—Solutions of the wild type α subunit or mixtures of mutant α subunits were concentrated, when this was required, by the addition of 0.43 g of solid ammonium sulfate per ml of solution (24). The salt was dissolved with stirring and the solution was allowed to stand 10 min at 0°. The suspension was then centrifuged at 27,000 × g for 10 min in the cold. The pellet was drained well and dissolved in an appropriate amount of 0.1 M KPO₄ buffer, pH 7.0, containing dithiothreitol. The concentration of dithiothreitol in this buffer and the subsequent dialysis was sufficient to give a 5-fold molar excess of dithiothreitol over the cysteine—SH concentration of the α subunit preparation used and was never less than 0.2 M. An excess of dithiothreitol was found to be necessary to diminish precipitation of the protein during the course of the subsequent urea treatment and dialysis. After the protein pellet had been dissolved in buffer, an aliquot was removed and diluted for subsequent assay. Sufficient solid urea was then added to make the solution 6 M with respect to urea. After standing for 40 min at 0°, the urea solution was dialyzed for 10 to 16 hours against 1 liter of 0.1 M KPO₄ (pH 7.0) containing dithiothreitol.

This entire procedure is referred to as “urea treatment and dialysis.” The small amount of precipitate that usually formed during dialysis was removed by centrifugation and the volume of the dialyzed protein solution was determined. Since relatively high concentrations of purified α subunit were required for dimer formation, it was desirable to use as small a volume as possible. A volume of 0.25 ml after the addition of urea was found to be the smallest practical working volume.

RESULTS AND DISCUSSION

Methods of Identifying and Determining Dimers Quantitatively

Separation from a Monomer by Size—Fig. 1A shows the elution profile obtained when a solution of highly purified α monomer was treated with urea and dialyzed as described under “Experimental Procedures” and chromatographed on a column of Sephadex G-100. Two peaks of indole to tryptophan activity are present; the smaller peak, eluting first, represents dimer while the much larger peak represents residual monomer. The activity associated with the material in the dimer peak is about 2% of that associated with the residual monomer peak. As is shown below, the indole to tryptophan activity associated with monomer and dimer fractions provides an accurate measure of the relative molar amounts of active species in these fractions. It can also be seen that there are three protein peaks, one associated with the dimer, one with the monomer, and one with material which appears at the void volume of the column. (In many experiments, a peak of indole to tryptophan activity is also associated with this last fraction (compare Fig. 5A).) Since the α subunit preparation used in this particular experiment had maximum specific activity, the appearance of significant amounts of protein at the void volume of the column indicates that considerable aggregation of α subunits occurs concomitant with the refolding which takes place during dialysis (compare protein profile with that of Fig. 1B).

Fig. 1B presents the control for the experiment in Fig. 1A. A sample of wild type α monomer from the same batch used in the previous experiment was treated as in Fig. 1A except that urea treatment was omitted. As can be seen, there is a single peak of indole to tryptophan activity, representing the α monomer, which corresponds to the single major protein peak. The control experiment shows that dimer active in the indole to tryptophan reaction is not present in preparations of the monomeric α subunit. In previous studies it was shown that aggregates of α monomer cannot be detected in the analytical ultracentrifuge, at low or high protein concentrations (11).

Formation and Detection of Enzymatically Active Dimers

The amount of dimer in a preparation is more conveniently assayed by a different method, which, however, is suitable only for complementing dimers. Table I presents results which show complementation in vitro of the mutant α subunits α₃₃ and α₄₆, and which show that complementation occurs only when the mutant α monomers are exposed together to 6 M urea. Although there is generally a significant loss of the input indole to trypto-

TABLE I

<table>
<thead>
<tr>
<th>Sample</th>
<th>Before urea treatment</th>
<th>After urea treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>α₃₃</td>
<td>3.0</td>
<td>2.4</td>
</tr>
<tr>
<td>α₄₆</td>
<td>3.6</td>
<td>3.4</td>
</tr>
<tr>
<td>α₃₃ + α₄₆, treated separately</td>
<td>3.3</td>
<td>2.8</td>
</tr>
<tr>
<td>α₃₃ + α₄₆, treated together</td>
<td>2.3₄</td>
<td>31.3</td>
</tr>
</tbody>
</table>

* α₃₃ and α₄₆ treated separately with urea and dialyzed and then mixed.

α₃₃ plus α₄₆ treated together with urea and dialyzed.

Sixty-four per cent of the input indole to tryptophan activity and 850% of the input InGP to indole activity were recovered after urea treatment and dialysis.

Protein preparations different from those used for Lines 1 to 3 were used in this case.
Phen activity following urea treatment, a net gain in InGP to indole activity is always observed (see Footnote c to Table I). Because of this irreversible loss of variable amounts of activity during renaturation from urea solutions, we have adopted the convention of normalizing the activity in the InGP to indole reaction to the activity recovered in the indole to tryptophan reaction. Other experiments have shown that the low yield of active \( \alpha \) chain dimers found under these conditions does not reflect the efficient complementation of a minority species present in each monomer preparation. It would thus appear that dimer formation under these conditions is inherently inefficient.

The elution profile from a Sephadex G-100 column of a 1:1 mixture of \( \alpha 33 \) and \( \alpha 46 \) treated together with urea is shown in Fig. 2. There are two peaks of indole to tryptophan activity. The first occurs at the position expected for an \( \alpha \) chain dimer. The second peak, which contains approximately 98% of the indole to tryptophan activity recovered from the column, is at the characteristic position of the \( \alpha \) monomer. In contrast, about 85% of the InGP to indole activity recovered is associated with the dimer peak. The values for the activity of each fraction in complementation units range from a high of 1000 for the dimer peak before and to a low of 3 for the monomer peak. The latter value is the same as that of the mixture of \( \alpha 33 \) and \( \alpha 46 \) before urea treatment. It is thus apparent that the entire increase in InGP to indole activity observed after urea treatment and dialysis is associated with one or more species larger than the monomer. The shoulder on the leading edge of the InGP to indole activity peak suggests that more than one species is responsible for the increase in activity. The amount of this larger material has varied in different experiments, comprising up to 50% of the indole to tryptophan activity which is not monomer (compare Fig. 5A). This material appears to be very heterogeneous and is rather unstable; at least some of it appears to dissociate to form dimer. It has not been investigated in detail. It would appear from Fig. 2, however, that there is a single major species, as defined by size, which has the majority of the InGP to indole activity which is detectable after urea treatment of an appropriate mixture of mutant monomers. We refer to this species as a dimer without any implication that it is a single, homogeneous species. A dimer composed of different mutant monomers, such as \( \alpha 33 \times \alpha 46 \), is referred to as a heterologous dimer, while one composed of identical monomers, such as \( \alpha 33 \times \alpha 33 \), is referred to as a homologous dimer.

As can be seen from Table I and Fig. 2, some mutant \( \alpha \) monomers have low but detectable levels of InGP to indole activity; the ratio of InGP to indole activity to indole to tryptophan activity for different mutant \( \alpha \) subunits varies from 1% to less than 0.001% of that of the wild type \( \alpha \) subunit. Occasionally this ratio varies by as much as 10-fold from batch to batch of a single mutant \( \alpha \) subunit. The cause of this variation is not known. It is not a serious problem, however, because the complementation unit reflects a change in the ratio of InGP to indole to tryptophan activity following urea treatment. The basal InGP to indole activity of mutant monomers is a problem, however, when attempting to determine the relative InGP to indole activities of complementing dimers and their parental monomers. Since the heterologous dimers constitute such a small fraction of the active species in a urea-treated preparation of \( \alpha \) monomers, this comparison cannot be accurately made in such a preparation if the monomer has appreciable basal activity. The magnitude of the inaccuracy can be best appreciated by comparing the ratio of complementation units before and after urea treatment in an unfractonated mixture of monomer plus heterologous dimer, \( 31.3:2.3, \) or \( 13.6 \) (Line 4, Table I), and the ratio for the dimer after separation from monomer, 1000:23 or approximately 400. The latter of these two ratios is the valid one, and indicates that a molecule of \( \alpha 33 \times \alpha 46 \) dimer has 400 times as much InGP to indole activity per unit indole to tryptophan activity as does a mixture of the \( \alpha 33 \) and \( \alpha 46 \) monomers of which it is composed.

### Process of Dimer Formation

In order to gain insight into the mechanism by which active dimers are formed, experiments were performed in which a 1:1 mixture of \( \alpha 33 \) plus \( \alpha 46 \) was exposed to various conditions and the yield of active dimer was measured. In the first experiment, the effect of urea concentration and of the time and temperature of incubation in urea on the formation of active dimers was examined. The results are shown in Fig. 3. A sharp rise in the InGP to indole activity occurs as the urea concentration increases, an apparent maximum being reached at 6 M. The recovery of input indole to tryptophan activity decreases gradually as the urea concentration increases. Some of the activity which was lost can be accounted for by an insoluble precipitate that formed during the course of dialysis. However, most of the inactivated protein remains in solution and is heterogeneous in size, as can be seen by comparing the protein profiles in Fig. 1, A and B. Comparison of the protein mixture in 6 M urea kept at 0° for 15 hours with that maintained at 0° for 40 min (Fig. 3) indicates that very little additional active dimer is produced or active monomer lost during prolonged incubation. This suggests that the unfolding required for active dimer formation is relatively rapid, and is completed in 40 min plus whatever time of dialysis is required to reduce the urea concentration from 6 M to 2 to 3 M. This comparison also suggests that, under these conditions, reaction of lysine residues with cyanate ions in the
Fig. 3. Generation of heterologous dimer and recovery of input monomer as a function of urea concentration and time and temperature of incubation in urea. Aliquots, 0.20 ml, of a 1:1 mixture of α33 plus α46 (10,000 units per ml) were added to various concentrations of urea, incubated for 40 min at 0°, and dialyzed overnight against 0.1 M KPO4 (pH 7.0), 1 mM dithiothreitol. Increase in complementation units (■) and recovery of initial indole to tryptophan activity (○) were then determined. Additional aliquots of the same α33 plus α46 mixture were made 6 M with respect to urea. One was incubated for 40 min at 37° and dialyzed. Increase in complementation units (▲) and recovery of indole to tryptophan activity (△) were measured. Another aliquot was incubated for 15 hours at 0° and dialyzed. Increase in complementation units (■) and recovery of indole to tryptophan activity (□) were measured. A third aliquot was diluted 1:10 with buffer and dialyzed. Increase in complementation units (×) and recovery of indole to tryptophan activity (●) were measured.

Fig. 4. Efficiency of dimer formation as a function of input monomer concentration. In two experiments, 1:1 mixtures of α33 plus α46 at concentrations ranging from 200 to 46,000 units per ml (0.08 to 18.4 mg per ml of total protein) were treated with urea and dialyzed. In each case, the α33 plus α46 preparation was 50% pure. The increase in complementation units was measured for each monomer concentration. Experiment 1, O; Experiment 2, ●.

urea solution (34) is not a major cause of inactivation of the α subunit. Other experiments support this interpretation and have shown that the specific activity of the purified residual monomer recovered after urea treatment and dialysis is the same as that of the input monomer. When the α33 plus α46 mixture in 6 M urea is incubated at 37° for 40 min, a considerable reduction is seen in both the amount of active dimer and the amount of active monomer recovered (Fig. 3). Under these conditions aggregation is greatly increased, as judged from the amount of precipitate formed.

Ten-fold dilution of the mixture in 6 M urea results in the lowest yield of active dimer and in the greatest recovery of input monomer. The high recovery of active monomers shows that irreversible modification of the monomers is not the cause of the low yield of dimers in this case.

Since a bimolecular reaction is required to form a dimer, the rate of dimer formation should be proportional to the square of the α subunit concentration; thus the opportunities for interchain contact and presumably dimer formation will be rapidly reduced by any process, such as dilution, which decreases the α subunit concentration. Conversely, intrachain interactions, leading to renaturation of monomers, should be favored relative to interchain interactions at low monomer concentrations. To test whether the formation of α chain dimers is a simple bimolecular reaction, 1:1 mixtures of α33 plus α46 at different concentrations were treated with urea and assayed. The combined results of two separate experiments are presented in Fig. 4. As can be seen, dimer formation increases very rapidly as monomer concentration is increased, reaches a broad maximum, and gradually decreases to about 50% of the maximum value at monomer concentrations of 45,000 to 50,000 units per ml. These data are clearly incompatible with expectations for a simple bimolecular reaction. The primary complicating factor seems to be the formation of soluble and insoluble aggregates of the α chain as the monomer concentration is increased. The recovery of input indole to tryptophan activity steadily decreases as the initial monomer concentration is increased, dropping from about 90% at the lowest monomer concentration to about 60% at the highest.

Gel filtration of preparations treated with urea at different monomer concentrations showed that the proportion of the input protein that appeared at the void volume of the column was significantly increased as the input monomer concentration was increased. It is perhaps not surprising that an increasingly large fraction of the urea-denatured monomer appears after dialysis as higher order aggregates as the initial monomer concentration is raised, since the probability of an n-monomer interaction increases in proportion to the nth power of the monomer concentration. Thus at high monomer concentrations, the probability of monomers interacting to form a large aggregate is very much higher per bimolecular interaction than at low monomer concentrations. It would not be expected, therefore, that the increase in dimer would be proportional to the square of the monomer concentration.

Formation of α Chain Dimers by Other Methods

Since the yield of dimer was low under optimal conditions of urea treatment, more efficient methods of dimer formation were sought. Crestfield, Stein, and Moore (35) have reported that RNase A will form dimers in 80% yield upon lyophilization from 50% acetic acid. When this procedure was attempted with wild type α monomer, an almost insoluble product retaining only 1% of its initial enzymatic activity was obtained. Guanidine hydrochloride has also been used to denature proteins (36). When a 1:1 mixture of α33 plus α46 was treated with guanidine hydrochloride in a manner analogous to our urea treatment and dialysis procedure, an increase of 31 complementation units occurred. This increase was accompanied by a recovery of 62% of the initial indole to tryptophan activity. These values are typical of, but no greater than, those obtained when the same
protein mixture is treated with urea (Table I). Substituting 0.1 M Tris-HCl (pH 7.8) for 0.1 M KPO₄ (pH 7.0) also fails to increase the yield of dimer. The effect of heating on dimer formation was also examined, since, as is described below, heating causes dissociation of the dimer into its constituent monomers. If this reaction were reversible dimers might be produced. To increase the likelihood of detecting heat stimulation of dimer production, heat-stable monomers were used in these experiments. A variety of conditions was tested, but dimer formation could not be detected.

**Stability of α Chain Dimers**

In view of the extended period of time required for some operations involved in the purification of dimer, it was necessary to examine dimer stability. Dimer preparations consistently lost activity when stored frozen while they were quite stable when stored unfrozen at 0°C; at 0-4°C, no more than 2 to 3% of purified dimer dissociated to monomer in a 5-day period. At 37°C, the α33α6 dimer was stable for at least 40 min when the components of the InGP to indole assay mixture were present. The enzymatic activity of dimer preparations did not survive ammonium sulfate precipitation. Therefore, dimer preparations were concentrated by dialysis against concentrated sucrose solutions, in which case 90% or more of the initial activity was recovered.

**Dissociation of α Chain Dimers**

Preliminary experiments indicated that, at 51°C, α chain dimers dissociate into their constituent monomers. In order to examine this dissociation in more detail, the homologous dimer α33α33 was prepared by urea treatment of the heat-stable monomer α33, and was purified by gel filtration.

As can be seen in Fig. 5A, the enzymatic activity was distributed among three well defined peaks. Approximately 2.0% of the indole to tryptophan activity was associated with large aggregates appearing near the void volume of the column. The dimer peak also contained 2.0% of the activity. The remaining 96% was associated with the monomer peak.

The specific activities of the active material in the peak fraction from each of the three peaks were 470 for the material near the void volume, 650 for the dimer, and 3800 for the monomer. Since the value for the monomer is the same as that of the starting material (before urea treatment), and since the amount of precipitate formed in this experiment accounted for only a small fraction of the 20% loss of indole to tryptophan activity during urea treatment of α33 (see legend, Fig. 5A), most of the protein corresponding to the 20% activity loss must appear in the fractions collected between the void volume of the column and the elution volume of the α33 monomer (compare protein profiles in Fig. 1, A and B). Thus fractions obtained from this portion of the elution profile will contain inactive protein and the specific activities determined for them will underestimate the true specific activity of the active species. It is shown below that most of the inactive protein in these fractions is derived from the α33 chain.

The fractions comprising the dimer peak from the above experiment were pooled, concentrated, and rechromatographed on the same column. The elution profile of this material is shown in Fig. 5B. As can be seen, the fractions pooled from the previous run contained only a small amount of monomer. The small shoulder on the leading edge of the dimer peak is presumably due to a slight contamination of the pooled fractions with the very large active species previously noted. The fractions containing

**Fig. 5. Purification and thermal dissociation of α33α33.** A, 38 mg of α33, specific activity 3700, were treated with urea and dialyzed at a protein concentration of 15 mg per ml. Of the initial indole to tryptophan activity, 80% was recovered. After addition of 4 mg of blue dextran 2000, the dialyzed protein was eluted from a column of Sephadex G-100 superfine, 1 x 100 cm, with 0.1 M KPO₄ (pH 7.0). 1 ml was dialyzed against a flow rate of 4 ml per hour. Of the activity applied to the column, 90% was recovered. B, the fractions corresponding to elution volumes between 37 and 44 ml in A were pooled, concentrated, and rechromatographed as in A. C, a portion of an α33α33 preparation, homogeneous by size and consisting of fractions corresponding to elution volumes between 37 and 44 ml in B, was heated at 51°C for 4 hours. It was then chilled and chromatographed as in A. D, as a control, another portion of the α33α33 preparation used in C was not heated. It was also chromatographed as in A after the chromatography of the heated preparation. Indole to tryptophan activity, •; indole to tryptophan activity on a condensed scale, ○. In all panels the void volume of the column is indicated by a vertical arrow.

**Fig. 6. Kinetics of increase in indole to tryptophan activity of a solution of α33α33 heated as described for 4 hours at 51°C.** The α33α33 preparation had 184 units per ml before heating and 291 units per ml after heating. The α33 preparation had 281 units per ml before heating and 290 units per ml after heating. The fraction of the initial indole to tryptophan activity for the α33α33 (○) and α33 (○) preparations is plotted on the ordinate. The solid line represents the increase in indole to tryptophan activity expected from enzymatically active α33α33 if (a) 50% of the α33α33 dissociates to 2 α33 following first order kinetics with a half-life of 45 min, (b) if 50% is nondissociable under these conditions, and (c) if 17% of the activity increase observed is due to the rapid conversion of an inactive species to an active one. See text for a discussion of the assumptions used in calculating the solid line.
the 37th to the 41th ml of eluate were pooled and used as the source of a33cr33 in subsequent experiments.

When this preparation of purified a33cr33 was heated at 51° and rechromatographed, a decrease in the size of the dimer peak and an increase in the amount of monomer were noted (Fig. 5C). In order to rule out the possibility that the monomer resulted from spontaneous dissociation of a33cr33 during the period required to perform this experiment, a portion of the purified a33cr33 preparation which had been stored at 0° was also rechromatographed (Fig. 5D). At most, 2 to 3% of the dimer dissociated to monomer over a period of 4 days at 0–4°. Therefore, the heating at 51° is responsible for conversion of dimer to monomer.

During the course of heating the a33cr33 preparation, aliquots were periodically removed and assayed for indole to tryptophan activity. The results of these assays are plotted in Fig. 6, where the unexpected finding is seen that the indole to tryptophan activity of the heated preparation increases as the dimer dissociates to monomer (Fig. 5C). The activity of a preparation of the a33 monomer, at a similar concentration, remained constant throughout the period of heating (Fig. 6). There are several plausible interpretations of the observed increase in indole to tryptophan activity of the heated a33cr33 preparation. They are: (a) that the a33cr33 dimer has a markedly lower affinity for \( \beta_t \) than does the a33 monomer, (b) that the a33–\( \beta_t \) complex has greater indole to tryptophan activity per active site than does the a33cr33 dimer–\( \beta \)-complex; (c) that the a33cr33 dimer, like the a33 monomer, has only a single \( \beta_2 \) binding site accessible to \( \beta_2 \) per molecule, and dissociation to monomer thus doubles the number of \( \beta_2 \) binding sites. Experiments described below have ruled out the first two alternatives and confirmed the third.

There are, however, several initially puzzling features about this series of experiments. First, it is clear from Fig. 5C that after 4 hours of heating a substantial amount of dimer remains as dimer, yet Fig. 6 shows that the increase in activity has virtually stopped by 4 hours. This suggests that there may be two different types of a33cr33 dimer, one which is heat-labile and the other heat-stable under these conditions. Second, it is possible to calculate the distribution of monomer and dimer from the increase in indole to tryptophan activity found at the end of the experiment, 1.58-fold. When this calculated distribution is compared to the observed distribution determined by summing the activities under the dimer and monomer peaks seen in Fig. 5C, the two do not agree. Third, expansion on the abacasa of the initial portion of the data in Fig. 6 indicates that the appearance of increased levels of indole to tryptophan activity during heating may be biphasic. This suggests that there may be, in addition to a heat-stable species, two forms of heat-labile species in the a33cr33 preparation used. These complexities can all be resolved if it is assumed that the a33cr33 preparation contains three species which have indole to tryptophan activity before or after heating. The fraction which is enzymatically active prior to heating appears to consist of a 1:1 mixture of different species of a33cr33 dimers, one species which does not dissociate detectably at 51° and the other which does so with first order kinetics, with a half-life of 45 min (solid line, Fig. 6). Crestfield and Fruchter (4) have shown that RNase A dimers dissociate with first order kinetics when heated. Two forms of RNase dimers have also been observed. However, when heated they dissociate into monomers at identical rates (2). It also appears that there is present in the a33cr33 preparation prior to heating a species which lacks indole to tryptophan activity but which is rapidly converted to active a33 monomer (with a half-life of less than 2 to 3 min) at 51°. It is this last species which would account for the initial rapid increase in activity at 51° (approximately 17% of the total increase observed) (Fig. 6) and which would introduce an error into calculations based on the assumption that the entire increase in indole to tryptophan activity observed after heating is due to dissociation of active a33cr33.

The distribution and absolute amounts of monomer and dimer seen in Fig. 5C coincide closely with those predicted if the species distribution described above for the a33cr33 preparation is assumed. Additional support for this interpretation comes from an experiment in which the a33cr33 dimer recovered after heating (first peak, Fig. 5C) was again heated. The rate of dissociation of this material, as judged from the rate of increase in indole to tryptophan activity at 51°, was only 30% of that observed in Fig. 6. Thus the a33cr33 dimer recovered from a once heated preparation is enriched for heat-stable species. However, an unequivocal demonstration of the existence of the multiple species postulated here must await their separation and characterization.

Preparations of dimers other than a33cr33 have also been observed to dissociate at 51°. The homologous dimer a46cr46 behaves similarly to a33cr33. The dissociation of the heterologous dimer a33dr33 results in an increase in indole to tryptophan activity coupled with the expected decrease in InGP to indole activity associated with the reconstituted active site. In other experiments, it was shown that \( \beta_2 \) stabilizes the a33cr33 dimer against heat dissociation.

The specific activity of the a33cr33 dimer preparation used in Figs. 5 and 6 was 650. The maximum specific activity of a33 is 5000. If a33cr33 has only a single binding site available to \( \beta_t \) per dimer, and, if it fully activates one of the two active sites on \( \beta_2 \), its maximum specific activity should be 2500. The fact that a lower specific activity is observed indicates either that inactive protein is present in the a33cr33 preparation or that the dimer is less effective than the monomer in activating \( \beta_2 \). To determine whether most of the inactive protein is a33 in some form, the a33cr33 dimer preparation was treated with 6 M urea at a low protein concentration (at which aggregation should be much reduced). Under these conditions, inactive a33 or aggregates thereof might be converted to active a33 monomers upon removal of the urea. Conversion of the active a33cr33 to 2 a33 would also be expected. It can be seen in Table II that, although an 11% loss in indole to tryptophan activity was observed in the control a33 monomer preparation, the a33cr33 sample showed a 575% gain in activity. This approximately 6-fold increase in activity is precisely what one would expect if most of the protein in the a33cr33 preparation were a33 in some form. The specific activity of the dimer preparation was 650, and 6 × 650 = 3900, a value reasonably close to the maximum specific activity of 5000. It is shown below that the active species in the a33cr33 preparation are as effective as the a33 monomer in activating \( \beta_2 \) per accessible \( \beta_2 \) binding site. Thus, most of the inactive protein in the a33cr33 preparation is the a33 chain in some form. Other data, discussed previously (see Figs. 5 and 6), suggest that there are several distinguishable species composed of a33 chains in the purified dimer preparation. Thus, one must conclude that renaturation of the a subunit from 6 M urea is an extremely complex process and that a heterogeneous population of aggregates, and possibly conformationally distorted monomers, results.
Sucrose Gradient Sedimentation Analysis of \( \alpha \) Chain Dimers

The sedimentation of \( \alpha 33\alpha 33 \), prepared and purified in a manner similar to that previously described, is shown in Fig. 7A. \( \alpha 33\alpha 33 \) sediments as a single symmetrical peak having a sedimentation coefficient of 3.7 S. Other \( \alpha \) chain dimers were similarly examined, including the \( \alpha 33\alpha 36 \) heterologous dimer. Since this dimer has InGP to indole activity, it can be detected when it is in the presence of large excesses of the parent monomers. The \( \alpha 33\alpha 36 \) dimer formed from a 1:1 mixture of \( \alpha 33 \) plus \( \alpha 46 \) after urea treatment has a sedimentation coefficient of 3.8 S to 3.7 S; when purified free of all monomer this dimer has the same sedimentation coefficient. The \( \alpha 33\alpha 36 \) dimer formed after treatment of \( \alpha 33 \) plus \( \alpha 46 \) with guanidine hydrochloride also has a sedimentation coefficient of 3.6 S to 3.7 S and in this case, also, the S value does not change after purification.

The wild type \( \alpha 33\beta 3 \) complex formed in \( \beta 3 \) excess and the \( \alpha 33\beta 3 \) complex formed in \( \alpha 33 \) excess have sedimentation coefficients of 5.6 S to 5.8 S and 6.4 S, respectively. 

Fig. 7B shows that, in the presence of a 10-fold excess of \( \beta 3 \) units, the \( \alpha 33\beta 33 \) sediments with an apparent sedimentation coefficient of approximately 6.1 S, while the excess \( \beta 3 \) sediments at its normal position, 5.1 S. As can be seen, there is no \( \alpha 33\beta 33 \)-stimulated indole to tryptophan activity at 3.7 S, indicating that all of the active \( \alpha 33\beta 33 \) dimers are in the 6.1 S complex with \( \beta 3 \). The sedimentation coefficient of the complex suggests that it consists of a single \( \alpha 33\beta 33 \) bound to a single \( \beta 3 \). Thus it would appear that, even in the presence of a considerable excess of \( \beta 3 \) units, only a single \( \beta 3 \) binding site on \( \alpha 33\beta 33 \) is accessible to \( \beta 3 \). If this is the case and the 6.1 S species has the structure \( \alpha 33\beta 33 \), this species should have twice as much \( \beta 3 \)-stimulated activity (in the presence of excess \( \alpha 33 \)) as \( \alpha 33 \)-stimulated activity (in the presence of excess \( \beta 3 \)) when assayed in the indole to tryptophan reaction; that is, in the presence of an excess of \( \beta 3 \), 1 molecule of \( \alpha 33 \) monomer and 1 molecule of \( \alpha 33\beta 33 \) dimer should contribute equal activity because each can form only one active site with the \( \beta 3 \) subunit. Correspondingly, an \( \alpha 33\beta 33 \) complex should have equivalent units of indole to tryptophan activity when assayed separately with excess \( \alpha 33 \) or excess \( \beta 3 \).

In the experiment presented in Fig. 7C, the sample layered on the gradient had a 1.65:1.00 ratio of \( \beta 3 \)-stimulated to \( \alpha 33\beta 33 \)-stimulated indole to tryptophan activity; i.e., according to the above considerations, the sample should contain twice as many molecules of \( \alpha 33\beta 33 \) as \( \beta 3 \). It can be seen in the figure that only about 60% of the \( \alpha 33\beta 33 \) activity but all of the \( \beta 3 \) activity is in a complex with a sedimentation coefficient of 6.6 S. The ratio of \( \beta 3 \)-stimulated to \( \alpha 33\beta 33 \)-stimulated indole to tryptophan activity for the complex ranges from 1.7 to 2.0. These findings are consistent with the structure \( \alpha 33\beta 33 \). (In other experiments it has been shown that when a mixture of \( \alpha 33\beta 33 \) and \( \beta 3 \) in a 1:2 activity ratio was sedimented, a single peak

Table II

<table>
<thead>
<tr>
<th>Species</th>
<th>Units before area treatment</th>
<th>Units after area treatment</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \alpha 33 )</td>
<td>64</td>
<td>57</td>
<td>89</td>
</tr>
<tr>
<td>( \alpha 33\beta 33 )</td>
<td>48</td>
<td>276</td>
<td>575</td>
</tr>
</tbody>
</table>

The sedimentation patterns of \( \alpha 33\beta 33 \) and mixtures of \( \alpha 33\beta 33 \) and \( \beta 3 \) in urea solutions containing 0.1 M KPO₄ (pH 7.0), 0.04 M pyridoxal-P, 5 mM L-serine. \( \alpha 33\beta 33 \) and \( \beta 3 \) were incubated together for 10 min at 37° in the same buffer (11) and chilled, and 0.20 ml was layered on each 4.8-ml gradient. The ratios of \( \beta 3 \)-stimulated and \( \alpha 33\beta 33 \)-stimulated indole to tryptophan activity in the solutions layered on the gradients were: A, 0:1; B, 10:1; C, 1:0:4:1; D, 0:60:1. Sedimentation was at 4° at 50,000 rpm for 8 hours (A) or 10 hours, 30 min (B, C, and D). Fifty-three 5-drop fractions were collected. Alternate fractions were assayed between the bottom of the gradient and the fastest moving component shown and in no case was \( \alpha 33\beta 33 \) or \( \beta 3 \)-stimulated activity found. All other fractions were assayed. Sedimentation coefficients were determined relative to InGP synthetase (2.6 S), \( \alpha (2.7 S) \), or \( \beta 3 (5.1 S) \) (8, 12, 24). (Previous studies of InGP synthetase at high concentration in the analytical ultracentrifuge have indicated that the sedimentation coefficient was 3.3 S (37). This value was not corrected for concentration dependence, however. At the dilute concentrations used in these studies, the sedimentation coefficient of InGP synthetase was found to be 2.8 S when compared to several other well studied reference proteins.) The vertical arrows represent the positions expected for species with the sedimentation coefficients indicated. Indole to tryptophan activity attributable to \( \alpha 33\beta 33 \), \( \beta 3 \), or \( \alpha 33 \), with all of each activity was obtained at 6.4 S.) The small increase in sedimentation coefficient observed, 6.6 S (Fig. 7C), as compared to 6.1 S (Fig. 7B), probably indicates the presence of a small amount of the \( \alpha 33\beta 33 \) (\( \alpha 33\beta 33 \) complex (see below).

Fig. 7D, in which \( \alpha 33\beta 33 \) is in 1.65-fold activity excess over \( \beta 3 \), and therefore presumably in 3.9-fold molecular excess, appears to show the existence of a new species, \( \alpha 33\beta 33 \) \( \alpha 33\beta 33 \). There are two peaks of \( \alpha 33\beta 33 \)-stimulated indole to tryptophan activity, one at approximately 7.1 S and the other at 6.6 S. The ratio of \( \beta 3 \)-stimulated to \( \alpha 33\beta 33 \)-stimulated indole to tryptophan activity in Fractions 18 through 23 of Gradient D, that is, the fractions under the 7.1 S peak, averages 1.08, close to that predicted for an \( \alpha 33\beta 33 \) \( \alpha 33\beta 33 \) complex.

Study of the complexes formed between \( \alpha \) chain dimers and \( \beta 3 \) subunits by zone sedimentation allows some tentative conclusions to be drawn about the structure of both the \( \alpha \) chain dimer and the


\[\beta_2\] subunit. The fact that only a single \(\beta_2\) subunit is able to form a complex with an \(\alpha\) chain dimer suggests either that the \(\alpha\) chain dimer has only a single \(\beta_2\) binding site or, if it has two, that they are close enough together that binding of another \(\beta_2\) subunit to the other. The fact that two \(\alpha_3\beta_3\) dimers can bind to a single \(\beta_2\) subunit suggests that the \(\alpha\) binding sites on the \(\beta_2\) subunit are not immediately adjacent to one another. This conclusion is consistent with the results of studies of the interaction of the wild type \(\alpha\) and \(\beta_2\) subunits (8).

**Specific Activity of \(\alpha_3\beta_3\)**

Determinations of the specific activity of dimers purified to apparent size homogeneity on Sephadex G-100 columns always give values between 400 and 700. The expected specific activity of a pure dimer is 2500. The low values observed have been ascribed to the presence of substantial amounts of enzymatically inactive protein derived from the \(\alpha\) chain in all dimer preparations purified by size. Since the contaminating protein is enzymatically inactive, it probably does not bind to the \(\beta_2\) subunit, or does so with an affinity much less than that of the active dimer. Active \(\alpha_3\beta_3\) might thus be separated from inactive protein by joining it in complex with \(\beta_2\) and sedimenting the complex in a sucrose gradient. When this was done, the specific activity of the \(\alpha_3\beta_3\) which was in complex with \(\beta_2\) was 1700, a value which is approximately 70% of the theoretical maximum specific activity of \(\alpha_3\beta_3\). The specific activity of the \(\alpha_3\beta_3\) preparation before centrifugation was 550.

**Determination of Apparent Molecular Weight of \(\alpha\) Chain Dimers by Sephadex Gel Filtration**

The molecular weights of \(\alpha\) chain dimers were estimated by comparing their elution volumes from a Sephadex G-100 column to the elution volumes of proteins of known molecular weight (38). The apparent molecular weights of the \(\alpha_6\beta_6\) dimer and the wild type \(\alpha\) chain dimer, estimated in this way, were 69,000 and 67,000, respectively, significantly greater than the theoretical molecular weight of 58,000. These findings and the results of the zone sedimentation experiments suggest that \(\alpha\) chain dimers are significantly more ellipsoidal in shape than the \(\alpha\) monomer. It can be calculated (39) that the sedimentation coefficient of a rigid sphere of twice the molecular weight of the \(\alpha\) monomer would be 4.8 S. However, the sedimentation coefficient of all \(\alpha\) chain dimers tested was 3.6 S to 3.7 S.

**Affinity of \(\alpha\) Chain Dimers for \(\beta_2\)**

Previous studies (11, 40) have shown that an apparent association constant, \(K_A\), for the association reaction between \(\alpha\) and \(\beta_2\) subunits can be determined from the enzymatic activities of the subunits and complex. Fig. 8 presents the results of an experiment in which increasing amounts of wild type \(\alpha\), \(\alpha_3\), and purified \(\alpha_3\beta_3\) were added to a constant amount of \(\beta_2\) subunit and the results plotted as described by Murphy (40). The \(K_A\) values determined from the slopes of these plots are 2.9 (units per ml)\(^{-1}\) for wild type \(\alpha\), 1.3 (units per ml)\(^{-1}\) for \(\alpha_3\), and 0.7 (unit per ml)\(^{-1}\) for \(\alpha_3\beta_3\). The values for the \(K_A\) of wild type \(\alpha\) determined previously by somewhat different methods are 5.3 (units per ml)\(^{-1}\) (11) and 4.2 (units per ml)\(^{-1}\) (40), while \(\alpha_3\) has been reported to have a \(K_A\) of 5.0 (units per ml)\(^{-1}\) (40). We have consistently found the \(\alpha_3\) value to be significantly lower than the value for wild type \(\alpha\) when determined by varying either \(\alpha_3\) or \(\beta_2\). From the above results, it would appear that the affinity of the \(\beta_2\) binding site on \(\alpha_3\beta_3\) which is accessible to \(\beta_2\) has been reduced by a factor of 2. Alternatively, the \(\beta_2\) binding site may be unaltered and the lowered apparent affinity may be due to partial blocking of the second \(\alpha\) binding site on \(\beta_2\) by the \(\alpha\) chain dimer.

The results presented in Fig. 8 confirm also that an \(\alpha_3\beta_3\) \(\beta_2\) \(\alpha_3\beta_3\) complex can activate \(\beta_2\) to the same degree that \(\alpha_3\) and wild type \(\alpha\) can activate \(\beta_2\), and that each of the two binding sites on \(\beta_2\) subunits can be saturated with \(\alpha_3\beta_3\). An alternative to this interpretation is that only one such site is saturated with \(\alpha_3\beta_3\), but that \(\alpha_3\beta_3\) is able to stimulate the \(\beta_2\) subunit in the indole to tryptophan reaction to twice the extent of either \(\alpha_3\) or \(\alpha_3\). In view of the fact that 22 different types of mutant \(\alpha\) subunits examined by Murphy (40), none was able to stimulate \(\beta_2\) more effectively than the wild type \(\alpha\) subunit and only one did so less effectively, and in view of the previous evidence for the existence of \(\alpha_3\beta_3\) \(\beta_2\) \(\alpha_3\beta_3\) (Fig. 7D), this latter alternative is considered unlikely.

\[K_M\] for IndGP of Monomer and Dimer

The \(K_M\) for IndGP of the \(\alpha_6\beta_2\) and \(\alpha_3\beta_6\beta_2\) complexes in the presence and absence of 0.6 M salt-free hydroxylyamine was determined by standard techniques (41). The results are presented in Table III. The presence of hydroxylyamine, which causes a 30- to 40-fold increase in the IndGP to indole activity of both the \(\alpha_6\beta_2\) and the \(\alpha_3\beta_6\beta_2\) complexes, has no effect upon the \(K_M\) for
InGP of either species. It appears that the reconstituted active site of the α33x46 dimer has a $K_M$ for InGP only 4- to 5-fold greater than the native active site. It is, of course, impossible to measure the $K_M$ for InGP of the mutant monomers. However, one of them, α46, has been shown to have a greatly decreased ability to bind InGP (42).

Relative Activities of α Chain Dimers and Wild Type α Subunit

The wild type α subunit has a characteristic ratio of activities in the reactions which it is able to catalyze (15). The comparable ratios of activities were determined for the dimers αwtαwt, α33x46, α33x33, and α46x46. Each of the dimers used was purified to size homogeneity by at least two passages through a Sephadex G-100 or Sephadex G-100 superfine column.

The results in Table IV indicate that inclusion of the wild type monomer in a dimeric structure has relatively little effect upon the ratio of the rates at which the reactions of the tryptophan synthetase complex are catalyzed. There is some indication that activation of the active site or sites on the dimer by β1 becomes progressively less efficient as the degree of activation increases. Thus, in the InGP to indole reaction (performed in the absence of hydroxylamine), the slowest tryptophan synthetase reaction in which InGP participates, the relative rate obtained with the dimer per unit of indole to tryptophan activity is 91% that of the monomer. At the intermediate reaction rate represented by the InGP to tryptophan reaction, the dimer is 67% as effective as the monomer per unit of indole to tryptophan activity. At the highest reaction rate, in the InGP to indole reaction in the presence of hydroxylamine, the activity of the dimer relative to the monomer dropped to 61%. This may reflect less effective contact between the β2 subunit and the β2 binding site on the αwtαwt dimer than between the β2 subunit and α monomer. The decrease in affinity observed between β2 and α33x33 relative to α33 is consistent with this interpretation. The striking feature of the comparison of the relative activities of α chain monomer and dimer, however, is the fact that the wild type α chain dimer has 3.2 times as much InGP to indole activity as does the α monomer. This comparison was performed with the intention of determining whether the wild type α chain dimer has one or two active sites for the InGP indole reactions. The wild type α monomer has a single β2 binding site for activation of β2 per catalytic site for the InGP to indole reaction. Thus the level of InGP to indole activity per unit of indole to tryptophan activity for the α monomer will be characteristic of a 1:1 ratio of β2 binding sites to InGP to indole catalytic sites. From experiments similar to those illustrated in Fig. 7, the wild type α chain dimer is also known to have a single β2 binding site accessible to β2 per molecule. However, it is not known whether it has one or two catalytic sites. If its InGP to indole activity

<table>
<thead>
<tr>
<th>Species</th>
<th>0.6 M NaCl added</th>
<th>$K_M$</th>
</tr>
</thead>
<tbody>
<tr>
<td>α·β2</td>
<td>-</td>
<td>0.05 MM</td>
</tr>
<tr>
<td>α33x46·β2</td>
<td>+</td>
<td>0.06 MM</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.25 MM</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.25 MM</td>
</tr>
</tbody>
</table>

Table IV

Relative enzymatic activities of αwt and αwtαwt in reactions of tryptophan synthetase complex

All rates are normalized to a rate of 100 in the indole to tryptophan reaction.

<table>
<thead>
<tr>
<th>Component or complex</th>
<th>Indole to tryptophan activity in 0.6 M hydroxylamine</th>
<th>InGP to indole activity in 0.6 M hydroxylamine</th>
<th>Ratio of InGP to indole activity to indole to tryptophan activity as a percentage of wild type ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>αwtαwt</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>α33x46-β2</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>α33x33-β2</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>α46x46-β2</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>α33x33-β2 + α46x46-β2</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

* InGP concentration was 0.4 mM.

Table V

Relative activities of αwt, α33x46, α33x33, and α46x46 in reactions of tryptophan synthetase complex

All rates are normalized to a rate of 100 in the indole to tryptophan reaction. α33x46, α33x33, and α46x46 were purified by chromatography on Sephadex G-100.

Complex | Indole to tryptophan activity | InGP to indole activity in 0.6 M hydroxylamine | Ratio of InGP to indole activity to indole to tryptophan activity as a percentage of wild type ratio |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>α33x33-β2 + α46x46-β2</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

* InGP concentration was 0.1 mM. The hydroxylamine used in this experiment was neutralized with 5.0 n KOH and stimulated InGP to indole activity to a lesser extent than did salt-free hydroxylamine.

These values represent the lower limit of detectability under the assay conditions used. (in the absence of β2) per unit of indole to tryptophan activity were the same as that of the monomer, it could be concluded that the dimer had only a single functional active site per molecule. If, however, this activity ratio were found to be twice that of the monomer, it would indicate that both potential active sites were functional in the dimer. There are several possible explanations for the observation that the dimer has more than twice the activity of the monomer. One is that the active site of the dimer actually catalyzes the InGP to indole reaction at 1.6 or 3.2 times the rate of that of the monomer. In view of the fact that, in all of the other reactions examined, the InGP-active site of the dimer was uniformly less active than that of the monomer (Table IV), this explanation seems unlikely. A more plausible interpretation is that the preparation of wild type α chain dimer used in these experiments contained substantial amounts of α chain which was unable to join in complex with β2 and activate it in the indole to tryptophan reaction. This inactive α chain species might nevertheless retain functional active site regions for cata-
lyzing the $\beta_2$-independent reaction, the conversion of InGP to indole. The evidence presented previously showing the presence of inactive $\alpha^{33}$ chains in a purified $\alpha^{33}\alpha^{33}$ preparation supports this interpretation. Since the $\alpha^{33}\alpha^{33}$ and $\alpha^{33}\alpha^{46}$ preparations were prepared in the same manner and had similar specific activities, this interpretation seems reasonable. It therefore seems likely that there are ways in which the $\alpha$ subunit can refold or aggregate after having been denatured in urea which result in inactivation of the $\beta_2$ binding site while leaving some degree of catalytic capability. This in turn suggests that the $\beta_2$ binding site and the catalytic site are independent. The question of the number of active sites per $\alpha^{33}\alpha^{33}$ dimer molecules remains unanswered, however.

Table V gives the results of a similar comparison of the activities of the $\alpha$ monomer, the $\alpha^{33}\alpha^{46}$ heterologous dimer, and the $\alpha^{33}\alpha^{33}$ and $\alpha^{46}\alpha^{46}$ homologous dimers. It can be seen that the activities of the purified homologous dimers in the InGP to indole reaction are very low. The heterologous dimer has at least 150 to 200 times as much InGP to indole activity per unit of indole to tryptophan activity as is the native site of the wild type dimer. Therefore, the active site region of the heterologous dimer, which presumably consists of parts of two polypeptide chains, appears to be formed with sufficient precision to be capable of catalyzing the conversion of InGP to indole at a minimum of 50% the rate characteristic of the active site of the $\alpha$ monomer. In fact, the reconstituted active site of the heterologous dimer may well be equally as effective in the InGP to indole reaction as is the native active site. If the $\alpha$ chain dimer is a symmetrical molecule, as would appear to be the case for the analogous RNase A dimer (3), it would be expected to have two equivalent $\beta_2$ binding sites (not necessarily simultaneously accessible to $\beta_2$) and two equivalent active sites per molecule. If a heterologous dimer were formed from different mutant $\alpha$ chains, only one of the active sites would be expected to be functional. It was shown previously that, per $\alpha$ chain dimer molecule, only a single $\beta_2$ binding site is accessible to $\beta_2$. Therefore, if there are two $\beta_2$ binding sites with equal affinity for $\beta_2$ and two active sites, only one of which is functional, at any moment only one-half of all of the heterologous dimer molecules would express InGP to indole activity in the presence of excess $\beta_2$; that is, one-half of all potentially active heterologous dimer molecules would be in complex at their $\beta_2$ binding site which is specific for the inactive reconstituted active site. Thus, only one-quarter of the dimer molecules present in an $\alpha^{33}\alpha^{46}$ preparation (containing $\alpha^{33}\alpha^{33}$ and $\alpha^{46}\alpha^{46}$) would have InGP to indole activity while all would activate $\beta_2$ in the indole to tryptophan reaction to the same extent that the monomer does. Thus, an InGP to indole to tryptophan activity ratio equal to 25% that of wild type $\alpha$ would indicate that the functional reconstituted active site of the heterologous dimer is fully as efficient in catalyzing the InGP to indole reaction as is the native site of the wild type $\alpha$ monomer. On the other hand, if the inherent activity of the reconstituted active site in the heterologous dimer was only 50% that of the wild type active site, then each of the functional active sites would of necessity be activated. This would require that each heterologous dimer bind to $\beta_2$ at the $\beta_2$ binding site which activates the functional active site. It seems unlikely that this would occur if there were two equivalent functional $\beta_2$ binding sites per dimer molecule.

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In other studies it has been shown that the $\alpha^{33}\alpha^{46}$ dimer has appreciable InGP to tryptophan activity when complexed with $\beta_2$.

**Model for Formation and Structure of $\alpha$ Chain Dimers**

The evidence presented thus far has established that: (a) urea treatment and dialysis of the tryptophan synthetase $\alpha$ subunit result in the formation of dimers of the $\alpha$ chain; (b) the increase in InGP to indole activity observed after denaturation of a mixture of mutant $\alpha$ monomers is associated with and dependent upon the formation of $\alpha$ chain dimers; (c) $\alpha$ chain dimers do not differ grossly from the monomers of which they are formed with respect to their affinity for the $\beta_2$ subunit, the extent to which they activate the $\beta_2$ subunit in the indole to tryptophan reaction, or their ability to catalyze the various reactions of the tryptophan synthetase complex in conjunction with $\beta_2$ (in the case of $\alpha^{33}\alpha^{33}$); (d) the $K_4$ for InGP of the reconstituted active site of a heterologous dimer differs from the wild type active site by a factor of only 4 to 5; (e) over-all catalytic efficiency of the reconstituted active site is lower than that of the native active site by at least a factor of 2; (f) $\alpha$ chain dimers...
have a single $\beta$ binding site per molecule which is accessible to $\beta_2$; (g) a chain dimers appear to be significantly more ellipsoidal than are $\alpha$ monomers; (h) chain dimers are dissociable to $\alpha$ monomers by heating; and (i) there are several active and inactive a chain species in $\alpha$ chain dimer preparations which are homogeneous by size.

Although the actual composition of a solution of dimer purified to apparent size homogeneity is rather complex, so that it is misleading to refer to "the $a33a33$ dimer," much less to "a chain dimers" as though there were only a single species, the generalizations discussed previously about all dimer species thus far tested do seem to be warranted. An additional point to be noted is that in the one case in which the same type of dimer was prepared by different methods, the resulting dimer solutions were not grossly different.

To account for the observations listed above, a model is presented in which dimer formation is caused by exchange of terminal portions of the contributing monomer chains (Fig. 9). The model is very similar to that proposed by Fruchter and Crestfield for RNase (3). Two possibilities for reciprocal exchanges are pictured, which we designate NH$_2$-terminal donation (Dimer A in Fig. 9) and COOH-terminal donation (Dimer B in Fig. 9). It can be seen that, although both of the dimers produced would be symmetrical and the formation of both would involve a reciprocal exchange of termini, the structures of the dimers formed would be different. Either form of reciprocal exchange of mutant $\alpha$ chain termini results formally in the construction of one functional active site region and one doubly altered one. However, one might imagine that only one form of exchange would enable an active catalytic site to be reformed and that the other would lead to a heterologous dimer without a functional active site for InGP to indole activity. Another variation on this model is that the translocation of termini need not be reciprocal. Thus the NH$_2$-terminal portion of one of the monomers might be incorporated into the structure of the second monomer upon refolding such that the NH$_2$ terminus of the second monomer remained as a relatively free "tail." In this case one would expect four different heterologous dimers and four different homologous dimers to be formed from two different mutant monomers, assuming that both NH$_2$-terminal and COOH-terminal donation can occur. Only two of these eight species might have a functional active site for the InGP to indole reaction. In principle, it should be possible to determine whether donation of the NH$_2$-terminal portion of the $\alpha$-polypeptide chain or the COOH-terminal portion can lead to reconstitution of a functional active site region by determining whether isolated segments of the $\alpha$-polypeptide chain can complement appropriately selected mutant $\alpha$ monomers. Experiments bearing on this question are discussed in the accompanying paper (21).

The model can explain the presence of heat-stable and heat-labile species of $a33a33$ if we assume, as has been done in Fig. 9, that one type of dimer is heat-stable at 51° and the other is not. Fig. 9 suggests a possible mechanism for this difference in that the interactions between monomers which hold the dimer together could be less extensive and therefore more susceptible to thermal disruption in the case of NH$_2$-terminal donation than in the case of COOH-terminal donation.

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As per the requirements, I will now provide the list of references at the end of the text:

REFERENCES

42. Hardman, J. K., and Yanofsky, C., Science, 156, 1369 (1967).
The Formation and Properties of Dimers of the Tryptophan Synthetase α Subunit of *Escherichia coli*

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