Roles of Hydrogen Bonding Residues in the Interaction between the $\alpha$ and $\beta$ Subunits in the Tryptophan Synthase Complex

Asn-104 OF THE $\alpha$ SUBUNIT IS ESPECIALLY IMPORTANT*

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The interaction of the $\alpha$ subunit with the $\beta_2$ subunit of tryptophan synthase is known to be necessary for the activation of each subunit and for the catalytic efficiency of the $\alpha_2\beta_2$ complex. To elucidate the roles of hydrogen bonds in the interaction site between the $\alpha$ and $\beta$ subunits for subunit association, eight mutant $\alpha$ subunits at five hydrogen bonding residues (N104D, N104A, N108D, N108A, E134A, E135A, N157D, and N157A) were constructed, and the thermodynamic parameters of association with the $\beta$ subunit were obtained using a titration calorimeter. The N104D and N104A mutations remarkably decreased the stimulation activity, the association constant and the stimulation activities, the association enthalpy was relatively small, though the association constant and the stimulation association constants, and the association enthalpies. Although the association constant and the stimulation activities of E134A were reduced in the absence of salt, the change in the association enthalpy was relatively small, and the addition of salt could repair its defects. The substitutions at positions 135 and 157 did not affect the stimulation activity and decreased the Gibbs energy of association corresponding to the defect in 1 mol of hydrogen bond. The present results suggest that the $\alpha$ subunit which has a mutation at position 104 cannot fold into an intact conformation upon complex formation, resulting in reduced stimulation activities. The hydrogen bond with Asn-104, which is a conserved residue among 16 microorganisms, was especially important for $\alpha/\beta$ interaction and mutual activation.

Protein-protein interactions play a central role in many physiologically important reactions. The tryptophan synthase complex is an excellent model enzyme for exploring the molecular recognition mechanism in protein-protein interaction (1, 2). Bacterial tryptophan synthase is an $\alpha_2\beta_2$ complex. The separated $\alpha$ and $\beta$ subunits catalyze inherent reactions termed the $\alpha$ and $\beta$ reactions, respectively (3–5). When the $\alpha$ and $\beta$ subunits combine to form the $\alpha_2\beta_2$ complex, the enzymatic activity of each subunit is stimulated by 1 to 2 orders of magnitude. It has been reported that this activation is due to conformational changes in both subunits upon $\alpha/\beta$ subunit interaction (reviewed in Ref. 1).

Some intermolecular electrostatic interactions (hydrogen bonds and salt bridges) and hydrophobic (packing) interactions were observed in many strongly bound complexes (6–8). In particular, electrostatic interaction is thought to play a critical role in protein-protein interaction (8–11). We focused on hydrogen bonds in the interaction site between the $\alpha$ and $\beta$ subunits of tryptophan synthase, termed herein “$\alpha/\beta$ subunit hydrogen bonds.” A thermodynamic study of the association of proteins having mutations at interface residues is important to elucidate the mechanisms of the subunit interaction in the tryptophan synthase complex. Also, it should provide useful information for understanding the energetic contribution of the hydrogen bonds that stabilize the protein complex. The amino acid sequences of the $\alpha$ and $\beta$ subunits from <i>Escherichia coli</i> are highly similar to those from <i>Salmonella typhimurium</i> (12); therefore, the three-dimensional structures have been considered to be almost identical (13). Residues that form hydrogen bonds in the interaction site between the $\alpha$ and $\beta$ subunits were identified from the x-ray structure of the $\alpha_2\beta_2$ complex from <i>S. typhimurium</i> (14). Table I identifies the hydrogen bonding partner in the $\beta$ subunit for the $\alpha$ subunit residues and the percent conservation of each residue (12, 15–26). In this paper, eight mutant $\alpha$ subunits at five positions were constructed except for position 133, which forms a hydrogen bond at an atom in the main chain (Table I). The roles of hydrogen bonding residues in the subunit interaction were investigated by activity measurements and titration calorimetry of the association. We will discuss the contribution of electrostatic interaction and the roles of hydrogen bonding residues in the subunit association in the complex.

**EXPERIMENTAL PROCEDURES**

**Materials**

**Mutant $\alpha$ Subunits from <i>E. coli</i>—**Five $\alpha/\beta$ hydrogen bonding residues of the $\alpha$ subunit from <i>E. coli</i> were replaced by means of site-directed mutagenesis using synthetic oligonucleotides. The sequences of the template DNAs and the oligonucleotides are shown in Table II. Glu-134 and Glu-135 can be directly substituted with Ala by a single code mutagenesis (E134A and E135A). Asn residues at positions 104, 108, and 157 were substituted with Asp (N104D, N108D, and N157D) or Ala (N104A, N108A, and N157A). The eight mutant $\alpha$ subunits from <i>E. coli</i> were purified as described (27).

$\beta$ Subunit from <i>E. coli</i>—The $\beta$ subunits of tryptophan synthase from <i>E. coli</i> can be obtained as a dimer form ($\beta_2$). The <i>E. coli</i> strain SP974 transformed with plasmid pUC9BG-2 (a gift from Dr. Somerville, Purdue University, West Lafayette, IN) coding the wild-type $\beta$ subunit of tryptophan synthase from <i>E. coli</i> was grown (28), and the protein was purified as described (29).

**Methods**

**Protein Concentrations**—Protein concentrations of the wild-type and mutant $\alpha$ subunits were estimated from the absorbance at 278.5 nm, assuming $E_{\text{abs}}^{1\text{cm}} = 4.4$ (30, 31). The $\beta$ subunit was estimated from the absorbance at 280 nm, assuming $E_{\text{abs}}^{1\text{cm}} = 6.5$ (32).

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*This work was supported in part by fellowships from the Japan Society for the Promotion of Science for Japanese Junior Scientists (to K. H.) and by a grant-in-aid for special project research from the Ministry of Education, Science, and Culture of Japan (to K. Y.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The intersubunit hydrogen bonding residues in the α subunit

| Structure | Residue | αδASA (%) | αASA (%) | ΔASA (%) | Hydrogen-bonding part | Conservation | Partner in β subunit (distance) | Conservation |
|-----------|---------|-----------|----------|----------|-----------------------|-------------|-------------------------------|-------------|
| t         | N104    | 1.06      | 60.72    | 59.66    | Side chain ND2        | 100         | 292G; main chain O             | 100         |
|           | N108    | 15.94     | 66.29    | 50.35    | Side chain ND2        | 25          | 290A^c; main chain O           | A:13        |
| r         | V133    | 3.77      | 6.60     | 2.83     | Main chain N          | 25          | 19Q; side chain OE1            | 19          |
|           | E134    | 40.73     | 73.90    | 33.17    | Side chain OE1        | 75          | 19Q; side chain NE2            | 19          |
|           | E135    | 13.81     | 66.05    | 52.24    | Side chain OE1        | 81          | 15M; main chain N              | 13          |
|           | N157    | 20.40     | 91.23    | 70.83    | Side chain ND2        | 31          | 181Y; side chain OH            | 19          |

a Secondary structure locating the residue of the α subunit. t and r represent a turn structure and a random loop, respectively.
b The values of αδASA and αASA mean the accessible surface area (ASA) calculated for the residues in the complex form and in the α subunit monomer without the β subunit, respectively. The values of ΔASA are the difference between them, indicating the area that is buried in the protein interior due to complex formation.
c The percentage of conservation at each position among the α or β subunits from 16 organisms: E. coli (12), S. typhimurium (12), Klebsiella aerogenes (12), Vibrio Parahaemolyticus (15), Pseudomonas putida (16), Pseudomonas aeruginosa (17), Caulobacter crescentus (18), Breibacterium lactofermentum (19), Bacillus subtilis (20), Lactobacillus casei (21), Saccharomyces cerevisiae (22), Thermus thermophilus (23), Bacillus stearothermophilus (24), Haloferax volcanii (25), Methanococcus voltae (26), and Bacillus coagulans.
d The hydrogen bonding partner of Asn-108 is Ala-290 in the complex from S. typhimurium, whereas it is Glu-290 in the complex from E. coli.

### Table II

The sequences of the synthetic oligonucleotide and template M13-trpA DNAs used for the site-directed mutagenesis of the α subunit of tryptophan synthase from E. coli

| mutant | Desired mutation | DNA sequence^a | Template M13-trpA^b |
|--------|------------------|----------------|---------------------|
| N104D  | Asn→Asp         | -5'TGTATGCCAATCCTGGTG-3' | Wild type       |
|        | Asp→Ala         | TGTATGCCAATCCTGGTG | N104D             |
| N108D  | Asn→Asp         | -5'TGGTTTGAACAAAAGCC-3' | Wild type       |
|        | Asp→Ala         | TGGTTTGAACAAAAGCC | N108D             |
| E134A  | Glu→Ala         | -5'GCCAGTTGAAGAGTCCG-3' | Wild type       |
|        | Glu→Ala         | GCCAGTTGAAGAGTCCG | N104D             |
| E135A  | Glu→Ala         | -5'CCAGTTGAAGAGTCCG-3' | Wild type       |
|        | Glu→Ala         | CCAGTTGAAGAGTCCG | N104D             |
| N157D  | Asn→Asp         | -5'GCCCGCAATGGCCGG-3' | Wild type       |
|        | Asp→Ala         | GCCCGCAATGGCCGG | N104D             |

^a The DNA sequence coding a residue to be substituted and the mismatch nucleotide are represented by the underlined and bold letters, respectively.

### Isothermal Titration Calorimetry (ITC)

Measurements of the Association between the α and β Subunits—The apparent association constant (K_a) and association enthalpy (ΔH_a) of the α subunit with the β subunit were calorimetrically determined at 40 °C using an OMEGA titration calorimeter from MicroCal, Inc., as already described (29, 33). Prior to the experiments, the sample proteins were dialyzed against a 50 mM potassium phosphate buffer, pH 7.0, containing 0.1 mM dithiothreitol, 5 mM EDTA, and 0.2 mM pyridoxal 5'-phosphate. For the experiments in the presence of additional salt, 300 mM KCl was added to the buffer. The solution of the β subunits in a cell (0.015–0.04 mm, 1.3115 ml) was titrated with the solution of the α subunits (0.1–0.3 mm) using a 250-μl long needle injection syringe. The ΔH_a and K_a values were calculated using the ORIGIN computer program (MicroCal, Inc.).

The experimental data were analyzed using a single set of identical sites model, where two α subunit binding sites per β subunit were identical and were independent of each other. The association Gibbs energy (ΔG_a) and entropy (ΔS_a) can be calculated from experimental K_a and ΔH_a values using the following equations,

\[
\Delta G_a = -RT \ln K_a
\]

\[
\Delta S_a = (\Delta H_a - \Delta G_a)/T
\]

1 The abbreviation used is: ITC, isothermal titration calorimetry.

Enzyme Assay—The forward α reaction, which is the conversion of indole-3-glycerol phosphate to indole, was measured by changes in the absorption at 340 nm of NADH produced in the reaction coupled with glyceraldehyde-3-phosphate dehydrogenase (34). The β reaction, which is the formation of L-tryptophan from indole and L-serine, was assayed utilizing the difference in absorption between indole and L-tryptophan at 290 nm (35). 1 unit of activity in each reaction is the conversion of 0.1 μmol substrate to product in 20 min at 37 °C

The inherent activities of the α subunits in the forward α reaction were determined in the absence of the β2 subunit in 0.1 M Tris-HCl buffer, pH 8.0, at 37 °C. The stimulation activities in the forward α and β reactions were determined in the presence of a constant amount of the wild-type or mutant α subunits and various amounts of the β subunit. The assay conditions for the stimulation activities were 50 mM potassium phosphate buffer, pH 7.0, at 37 °C, the same conditions as used for the calorimetry, in the presence and absence of 300 mM KCl. For the stimulation activities in the β reaction, the inherent activities of the β subunit at various concentrations were measured and subtracted.

### RESULTS

**Stimulation Activities of the Mutant Tryptophan Synthase**—To determine the effect of the deletion of the hydrogen bonds on the stimulation activities, the five mutant α subunits having Ala at the intersubunit hydrogen bonding site (N104A,
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N108A, E134A, E135A, and N157A) were investigated. We also investigated three Asp mutant α subunits (N104D, N108D, and N157A) to determine the effect of the introduction of a charged residue at these positions. All the mutant enzymes should lack one hydrogen bond between the α and β subunits. These mutant α subunits retained the inherent activities in the forward α reaction (about 0.1 units/mg), indicating that the substitutions did not affect the monomer structure of the protein.

Fig. 1 shows the stimulation activities in the β reaction of several α subunits (wild type, N104D, N104A, N108D, N108A, and E134A) as a function of concentration of the β subunit. The other substitutions (E135A, N157D, and N157A) had little effect on the stimulation activities (data not shown). In the absence and presence of 300 mM KCl, the titration curves of N104D, N104A, and N108D α subunits were more gradual than that of the wild-type α subunit, indicating weaker association with the β subunit. The curve of N108A α subunit was sharp and similar to that of the wild-type, suggesting that the weakened association of N108D α subunit is not due to the lack of a hydrogen bond. In the absence of KCl, the titration curve of E134A α subunit was more gradual than that of the wild type, but the weakened association could be repaired by the addition of 300 mM KCl. In the forward α reaction, similar titration curves were found (data not shown). These results indicate that the hydrogen bonds, except for position 104, are not required for the mutual activation.

Titration Calorimetry of the Interaction of Mutant α Subunits with the β Subunit—The mutual activation between the α and β subunits is induced by the formation of the αβ complex. To determine the effect of deletion of a hydrogen bond on the αβ subunit interaction, the thermodynamic parameters of association of mutant α subunits with the β subunit were examined using ITC. Fig. 2 shows the calorimetric titration curves of the β subunit with the wild-type and mutant α subunits at 40 °C, pH 7.0. The titration curves of four mutant α subunits (N104D, N104A, N108D, and E134A) had a more gradual transition than that of the wild-type α subunit, indicating the decreases in the association constants ($K_A$) of these mutant α subunits. The thermodynamic parameters of association were calculated by the computer program ORIGIN (29, 33), assuming that the two α subunit binding sites of the β subunit were identical and independent of each other (i.e. single set of identical sites model), and are shown in Table III. The independent or interactive two sites model was also tried, but these models failed to
between the α subunits with the β subunit at 40°C in the absence and the presence of 300 mM KCl

All the molar concentrations for each parameter represent the molar concentration of the α chain. Experimental errors for $K_a$ and $\Delta H_a$ of the wild type are ± 0.95 × 10⁻⁹ M⁻¹ and ± 8.6 kJ mol⁻¹, respectively, which result in errors in $\Delta G_a$ of ± 0.5 kJ mol⁻¹ and $-T\Delta S_a$ of ± 7.9 kJ mol⁻¹ K⁻¹.

| α subunit | $K_a$ (10¹⁶/M) | $\Delta H_a$ (kJ/mol) | $\Delta G_a$ (kJ/mol) | $-T\Delta S_a$ (kJ/mol) | $-T\Delta S_{m,W}$ (kJ/mol) |
|-----------|----------------|----------------------|---------------------|----------------------|---------------------------|
| −KCl⁵ | 5.32 | -147.3 | -40.3 | 107.0 |
| N140D | 0.30 | -97.1 | 50.2 | -32.8 | 6.43 | -42.7 |
| N140A | 0.35 | -119.2 | 28.1 | -33.2 | 7.1 | -21.0 |
| N108D | 0.29 | -105.9 | 41.4 | -32.8 | 7.5 | -33.9 |
| N108A | 4.40 | -139.3 | 8.0 | -39.8 | 0.5 | 99.5 | -7.5 |
| E134A | 0.16 | -134.7 | 12.6 | -31.2 | 9.1 | 103.5 | -3.5 |
| E135A | 1.20 | -151.9 | 4.6 | -36.4 | 3.9 | 115.5 | 8.5 |
| N157D | 1.25 | -150.2 | 2.9 | -36.6 | 3.7 | 113.6 | 6.6 |
| N157A | 2.45 | -152.7 | 5.4 | -38.3 | 2.0 | 114.4 | 7.4 |

+KCl⁶ |
| Wild type | 11.05 | -118.8 | -42.2 | 76.6 |
| N104D | 0.19 | -62.3 | 56.5 | -31.6 | 10.6 | 30.7 | -45.9 |
| N104A | 0.22 | -96.2 | 22.6 | -32.0 | 10.2 | 64.2 | -12.4 |
| E134A | 2.50 | -135.1 | 16.3 | -38.4 | 3.8 | 96.7 | 20.1 |

⁵ Thermodynamic parameters were measured in 50 mM potassium phosphate buffer, pH 7.0, in the absence (−KCl) or presence (+KCl) of 300 mM KCl as described under "Experimental Procedures."

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fit the titration curves. All titration curves of the mutant enzymes demonstrate a similar stoichiometry of about 0.7 α chain/β chain. The departure from the expected 1:1 stoichiometry probably results from the presence of a fraction of inactive β subunit, which cannot bind the α subunit (29). The thermodynamic parameters of association are independent of the binding number because they are estimated by the molar concentration of the α subunit.

The negative $\Delta G_a$ values of almost all mutant α subunits were decreased compared with that of the wild-type α subunit. The changes in the $\Delta G_a$ varied with the substituted positions. The change in the $\Delta G_a$ of the N108A α subunit was the smallest among the mutant enzymes. However, there was a subtle change in the $\Delta H_a$ and it was compensated by a change in the $-T\Delta S_a$. The $\Delta G_a$ values for E135A, N157D, and N157A α subunits were affected by 2.0–3.9 kJ mol⁻¹, and these defects resulted from an unfavorable increase in the $-T\Delta S_a$ values. Remarkable changes were observed for N104D, N104A, N108D, and E134A α subunits. Their $K_a$ values were decreased by 1 order of magnitude, and the decreases in the negative $\Delta G_a$ values were 7.1–9.1 kJ mol⁻¹ compared with that of the wild-type α subunit. The negative $\Delta H_a$ values of N104D, N104A, and N108D α subunits were unfavourably decreased compared with that of the wild-type α subunit. The decrease in $\Delta H_a$ of the E134A α subunit was relatively smaller than that of the N104D, N104A, or N108D α subunits although the $K_a$ value of the E134A α subunit was similar to that of the others.

Effect of Salt on α/β Subunit Interaction—Electrostatic interactions have been reported to contribute to many intermolecular associations based on x-ray crystallographic analysis (8, 9), thermodynamic analysis (10), and computer simulation (11). To investigate the electrostatic contribution to the interaction between the α and β subunits, the effect of salt was examined by ITC. Fig. 3a shows the titration curve for the wild-type α subunit in the absence and presence of 300 mM KCl. The thermodynamic parameters of association are shown in Table III. The $K_a$ values showed that the complex formation of the wild-type tryptophan synthase was increased by the addition of KCl. The negative $\Delta H_a$ of the wild-type α subunit in the presence of KCl was lower than that in the absence of additional salt (Table III). However, the favorable increase in the $\Delta S_a$ overcame the unfavorable $\Delta H_a$ and resulted in a favorable decrease in $\Delta G_a$.

The effects of the substitutions at the interface on the electrostatic properties of association were then examined by ITC in the presence of 300 mM KCl for the mutant α subunits at positions 104 (N104D and N104A) and 134 (E134A), which have decreased $K_a$ values in the absence of salt (Fig. 3 and Table III). The negative $\Delta H_a$ values of N104D and N104A subunits were decreased by the addition of KCl in a way similar to that of the wild type, whereas the $K_a$ values were slightly decreased. In the case of E134A α subunit, the $K_a$ value was greatly increased by the addition of KCl. The negative $\Delta H_a$ value of E134A α subunit in the presence of KCl was larger than that of the wild type, in contrast with that in the absence of KCl.

**DISCUSSION**

The Electrostatic Repulsion between the α and β Subunits—Recently, it has been reported that monovalent cations change the conformation of the tryptophan synthase complex and activate it (36–41). The preferential binding of a cation (such as Na⁺ or K⁺) to a specific site in the β subunit has high affinity, and the site is saturated at 20 mM cation concentration (38). In this study, because we treated the sample in 50 mM potassium phosphate buffer, the concentration of K⁺ might be enough to saturate the cation binding sites of the tryptophan synthase complex even in the absence of additional KCl. Therefore, the increase in the $K_a$ value of the wild type on the addition of salt observed here (Table III) should not result from a conformational change due to the effect of the cation. Alternatively, the present results suggest the presence of electrostatic repulsion between the α and β subunits. The addition of salt might diminish this repulsion and cause an increase in the affinity. Similar stabilizing effects of salts on the oligomer structure have been reported for some proteins (42–47).

We examined whether there is an electrostatic repulsion between the α and β subunits due to the complex structure (14). The charges on the subunit interface were calculated using the Insight II-Delphi program (48), and it was found that both subunits were relatively negatively charged at the subunit interface. A qualitative estimation using the Insight II-Delphi program showed that the electrostatic effect acts unfavorably on the association between the α and β subunits, whereas the van der Waals interaction is favorable.

In the cases of the mutant α subunits at position 104, the presence of salt to shield the electrostatic repulsion did not improve the affinity for the β subunit (Table III). In contrast, the
weakened affinity of E134A α subunit was repaired by the presence of salt and was close to that of the wild type (Table III).

The Role of the Hydrogen Bond between the α and β Subunits in the Association—Shirley et al. (49) have reported that the contribution of a hydrogen bond to the Gibbs energy of unfolding is 5.4 ± 2.5 kJ mol⁻¹ using mutant proteins. Fersht (50) has also evaluated it to be 2.1–7.5 kJ mol⁻¹. In the present study, the average ∆ΔGₐ of eight mutant proteins was 5.2 kJ mol⁻¹ (Table III), and it fell within the ranges reported previously. However, the other thermodynamic parameters, ∆Hₐ and ∆Sₐ, varied with the substituted positions (Table III). This indicates that hydrogen bonds play different roles in the association depending on their positions (see below).

The Effects of the Deletions of the Hydrogen Bonds at Positions 135 and 157 Are Localized in Substituted Positions—The effects on the thermodynamic parameters of association of the mutant α subunits at positions 135 and 157 (E135A, N157D, and N157A) could be considered to be due to the deletion of only 1 mol of hydrogen bond (Table III). In each case, the reasonable decrease in the negative ∆Gₐ value corresponds to the deletion of one hydrogen bond as reported previously (49, 50). Although the changes in the ∆Hₐ and −T∆Sₐ of these mutant α subunits were mostly within experimental error, there is a tendency for the unfavorable ∆Gₐ to be accompanied by an unfavorable change in the positive −T∆Sₐ and partially compensated by a favorable change in the negative ∆Hₐ. These results agree with the conclusion of Privalov and Makhataze (51) that the hydrogen bonds between the polar groups are stabilized entropically. Connelly et al. (52) have studied the effect of the substitution of a hydrogen bonding residue on the protein-ligand binding using ITC and have found similar results. Because the changes observed for the substitutions at positions 135 and 157 correspond to the deletion of one hydrogen bond as described above, the effects due to the mutations must be localized in the substituted region.

The substitutions at positions 135 and 157 (E135A, N157D, and N157A) had no effect on the stimulation activity, and the changes in the thermodynamic parameters are in the ranges of the contribution from one hydrogen bond as described above (Table III), suggesting that these substitutions do not result in a defect in the intact conformation of the complex. These residues are located on the edge of the interface with the β subunit, far from the intersubunit tunnel, and the partner residues of the β subunit (Met-15 and Tyr-181 of the β subunit) are not highly conserved. Therefore, it can be concluded that these hydrogen bonds are not very important for the mutual activation.

The Hydrogen Bond at α Subunit Asn-104 Is Especially Important for Association With the β Subunit—Due to the substitutions at position 104 (N104D and N104A), the stimulation activities were decreased (Fig. 1), and the decreased Kₛ values of these mutant α subunits (Table III) might be related to the reduced stimulation activities. We have reported that the complex formation couples with the folding and the rearrangement events in the α or/and β subunits, and the folding might occur not only at the subunit contact surface but also at other parts in the molecules (53). The unfavorable decreased ∆Hₛ values of the mutant enzymes at position 104, compared with that of the wild type (Table III), might suggest that the mutant proteins cannot fold into an intact conformation upon complex formation. Therefore, the stimulation activities of their resulting complexes were lower than that of the wild-type complex. Furthermore, the affinities of N104D and N104A with the β subunit could not be improved by the addition of salt (to seal electorostatic repulsion) (Table III), suggesting that these mu-
tant α subunits could not associate with the β subunit to fold the similar conformation to the αβα complex of the wild-type protein.

Position 104 is apparently located far from the intersubunit tunnel. However, in the αββα complex form, the Asn residue at position 104 of the α subunit contacts the residue at position 292 of the β subunit in a sharp turn in the tryptophan-sensitive “hinge” region (Table I). This hinge region is included in the long stretch from 260–310 (with a loop conformation), which forms one side of the intersubunit tunnel of the β subunit (14).

The substitution (28, 54) or proteolysis (55–57) in the “hinge” region of the β subunit reduces the stimulation activity, the substrate affinity, and the association between the α and β subunits. Thus, this region is believed to play a critical role in the conformational change upon the subunit association (1). The present results revealed the special role of 104; the hydrogen bonding at position 104 is necessary to maintain the hinge of the β subunit in an intact form, and a substitution at this position causes a significant defect in the stimulation activity. Regarding other mutations at position 104 of the α subunit (58), it has also been reported that the N104S mutant decreases the activity in the αββα complex form, the Asn residue at position 104 is apparently located far from the intersubunit tunnel, and is replaced by Glu in E. coli in the hinge region of the β subunit. The extreme substitution with a bulkier and more polar residue at position 108 (N108D) might prevent intact conformation of the complex, but hydrogen bonding at position 108 is not required for complex formation as shown in results of N108A. Another mutation at this position (N108S) also does not affect the activity of the complex (58).

The ΔG0 value of E134A α subunit in the absence of KCl was remarkably affected by the substitution, although the change in the ΔH0 was relatively small (Table III). In the case of E134A α subunit, the conformational change due to the complex formation might correspond to that of the wild type, despite its low K0 value. This is also supported by the results in which the ΔG0 was improved by the addition of the KCl, and the negative ΔH0 value and positive ΔTS value could be explained by the deletion of one hydrogen bond as described for the substitutions at positions 135 and 157. Glu-134 is 40% exposed to the solvent even in the complex form (Table I). The percentage of conservation of Glu-134 is 75%, but its hydrogen bonding partner Gln-19 of the β subunit is not highly conserved (Table I).

Present results indicate that this residue is not required to induce complement association although the substitution of Glu-134 affected the affinity for the β subunit.

Conclusions—The hydrogen bonding residues located in the αβ subunit interface play various roles in the association and mutual activation, depending on their positions. The deletion of the hydrogen bond at Asn-104 affected the thermodynamic parameters of association with the β subunit and remarkably reduced the stimulation activity in the β reaction. It can be concluded that the hydrogen bond at Asn-104 of the α subunit is especially important for intact association with the β subunit and mutual activation of the complex.