Stimulated Interaction between α and β Subunits of Tryptophan Synthase from Hyperthermophile Enhances Its Thermal Stability*

Kyoko Ogasahara‡, Masami Ishida§, and Katsuhide Yutani¶

From the Institute for Protein Research, Osaka University, Suita City, Osaka 565-0871, the Institute for Protein Research, Osaka University, Graduate School of Sciences, Gakuen 2-1 Sanda City, Hyogo 667-1337, Japan

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Tryptophan synthase from hyperthermophile, Pyrococcus furiosus, was found to be a tetrameric form (αβ)2 composed of α and β subunits. To elucidate the relationship between the features of the subunit association and the thermal stability of the tryptophan synthase, the subunit association and thermal stability were examined by isothermal titration calorimetry and differential scanning calorimetry, respectively, in comparison with those of the counterpart from Escherichia coli. The association constants between the α and β subunits in the hyperthermophile protein were of the order of 108 M−1, which were higher by two orders of magnitude than those in the mesophile one. The negative values of the heat capacity change and enthalpy change upon the subunit association were much lower in the hyperthermophile protein than in the mesophile one, indicating that the conformational change of the hyperthermophile protein coupled to the subunit association is slight. The denaturation temperature of the α subunit from the hyperthermophile was enhanced by 17 °C due to the formation of the αβ2 complex. This increment in denaturation temperature due to complex formation could be quantitatively estimated by the increase in the association constant compared with that of the counterpart from E. coli.

Hyperthermophilic proteins, which retain the folded conformation and maximally express their function near the boiling point of water, have been the target of extensive studies on protein stabilization, folding, structure, and evolutionary aspects over the past decade. Much work has been done to determine the three-dimensional structures of hyperthermophile proteins and to identify the structural determinants of the enhanced stability. A comparison of the structures of proteins from hyperthermophiles with their mesophilic counterparts has led to a better understanding of several features of the hyperthermophile proteins (1, 2, 19–22). One of these is that several hyperthermophile proteins have structures with a higher degree of oligomerization compared with the mesophilic homologues. Triose phosphate isomerase from hyperthermophiles is found to be tetrameric in contrast to the dimeric form from mesophilic sources (3–6). Hyperthermophilic phosphoribosylanthranilate isomerase is dimeric, but the proteins from mesophilic organisms are monomeric (7). Hyperthermophilic lactate dehydrogenase exists as tetrameric or octameric forms (8). Moreover, extra ion pairs or hydrophobic interactions have often been found in the subunit/subunit interface of proteins from hyperthermophiles (9–18). On the bases of these observations, a hypothesis has been proposed that the higher order oligomerization of subunits and strong subunit association are potentially important for enhanced stability of hyperthermophile proteins (19–22). However, there are few studies that characterize the strength of the subunit association in the hyperthermophile proteins and quantitatively elucidate the correlation between the subunit association and stability. Elucidating the subunit association feature in hyperthermophile proteins is an important subject for understanding the mechanism of anomalous stability and of protein-protein recognition itself in oligomeric proteins. Isothermal titration calorimetry is a powerful method for thermodynamically assessing protein-protein interactions, which are especially useful for measuring association parameters. There has been little application of isothermal titration calorimetry to characterize subunit association in hyperthermophile proteins.

We are now focusing our attention on the subunit association in tryptophan synthase from the hyperthermophile, Pyrococcus furiosus, in connection with thermal stability. Prokaryotic tryptophan synthase (EC 4.2.1.20) with the subunit composition αβ2 is a multifunctional and allosteric enzyme. This αβ2 complex has an αβαβ arrangement (23) and can be isolated as the α monomer and β2. The α and β2 subunits catalyze inherent reactions (for reviews, see Refs. 24–28). When the α and β2 subunits associate to form the αβ2 complex, the enzymatic activity of each subunit is enhanced by 1 to 2 orders of magnitude (for reviews, see Refs. 24–28). The αβ subunits interaction is important for the mutual activation of the each subunit in prokaryotic tryptophan synthase. We found that tryptophan synthase (PTSase) from P. furiosus was also composed of αβ2, and the enzymatic activities of the α and β subunits separated in their active forms were stimulated by the formation of the αβ2 complexes as well as the reported mesophilic prokaryotic bacterial tryptophan synthase (for reviews, see Refs. 24–28). The thermal stability of the α subunit of PTSase is remarkably higher than that from Escherichia coli (29).

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†To whom correspondence should be addressed: Kwansei Gakuen University, Graduate School of Sciences, Gakuen 2-1 Sanda City, Hyogo 667-1337, Japan, Tel.: 81-795-65-8482, Fax: 81-795-65-9077, E-mail: yutani@ksc.kwansei.ac.jp.

1 The abbreviations used are: TSase, tryptophan synthase; PTSase, EcTSase and StTSase, tryptophan synthase from P. furiosus, E. coli and S. typhimurium, respectively; Pfu, Ecβ2, and Pfuαβαβ, α, β subunits and αβ2 complex of PTSase, respectively; Eco, Ecβ2, and Ecoαβαβ, α, β subunits and αβ2 complex of EcTSase, respectively; Stα, Stβ2, and Stαβ2, α, β subunits and αβ2 complex of StTSase, respectively; FTC, isothermal titration calorimetry; DSC, differential scanning calorimetry; PLP, pyridoxal 5’-phosphate; DT, dithiothreitol.
Tryptophan synthase from hyperthermophiles is an attractive model system for seeking correlation between subunit association and stability.

In this report, to elucidate the subunit interaction feature in PyTSase in connection with thermal stability and tryptophan synthase from E. coli (EcTSase), the subunit association and thermal stability were measured by isothermal titration calorimetry and differential scanning calorimetry, respectively. The results revealed that the binding between the α and β subunits in PyTSase was strong compared with that in EcTSase, leading to the enhanced stability of the protein and the high temperature adaptation of the tryptophan synthase function.

EXPERIMENTAL PROCEDURES

Expression and Purification of α, β, and αβ from P. furiosus—The α subunit (Pfα) from P. furiosus was expressed in the E. coli strain JM109/pBM1974 (30) and purified as described previously (29). Each of the genes of trpB and trpBA from P. furiosus was transformed into the E. coli strain JM109 (30). E. coli, harboring each of the genes, was grown in 15 liters of Luria-Bertani medium supplemented with ampicillin at 100 mg/liter culture medium at 37 °C. The expressions of trpB and trpBA were induced by isopropyl-β-D-thiogalactopyranoside added at a concentration of 1 mM to the culture medium 1 h after starting the culture. After culturing for 20 h, the cells were harvested and suspended in 100 mM of 20 mM potassium phosphate buffer (pH 7.0) containing 0.02 mM PLP, 5 mM EDTA, and 1 mM DTT. After sonication and heat treatment of the homogenized solution for 10 min at 75 °C, cell debris and denatured E. coli proteins were removed by centrifugation at 15,000 rpm for 30 min at 4 °C.

For Pβα, the precipitate with ammonium sulfate at 60% saturation was dissolved in 50 ml of 25 mM potassium phosphate buffer (pH 7.0) containing 0.02 mM PLP, 5 mM EDTA, and 1 mM DTT and dialyzed against the same buffer overnight at 4 °C. The dialyzed sample was applied on a column (2.5 × 27 cm) of DEAE-Sephaloc (Amersham Biosciences) and eluted with a linear gradient of 25 to 500 mM potassium phosphate buffer (pH 7.0) containing 5 mM EDTA and 1 mM DTT. The active fractions of the eluted solutions were concentrated and applied on a column (2.5 × 50 cm) of Sepharose 26/10 (Amersham Biosciences) with a linear gradient of 25 to 500 mM potassium phosphate buffer (pH 7.0) containing 0.02 mM PLP, 1 mM EDTA, and 5 mM DTT. After elution with a linear gradient of 25 to 500 mM potassium phosphate buffer (pH 7.0) containing 5 mM EDTA and 1 mM DTT, the active fractions of the eluted solutions were concentrated and applied on a column (2.5 × 27 cm) of DEAE-Sephaloc (Amersham Biosciences) and eluted with a linear gradient of 25 to 500 mM potassium phosphate buffer (pH 7.0) containing 5 mM EDTA and 1 mM DTT. The active fractions were then purified by ion exchange chromatography (Q Sepharose 26/10, Amersham Biosciences) with a linear gradient of 25 to 200 mM potassium phosphate buffer (pH 7.0) containing 5 mM EDTA and 1 mM DTT.

For Pfβαβ, the precipitate with ammonium sulfate at 60% saturation was dissolved in 50 ml of 10 mM potassium phosphate buffer (pH 7.0) containing 0.02 mM PLP, 5 mM EDTA, and 1 mM DTT and dialyzed against the same buffer overnight at 4 °C. The dialyzed sample was applied on a column (2.5 × 27 cm) of DEAE-Sephaloc (Amersham Biosciences) and eluted with a linear gradient of 25 to 500 mM potassium phosphate buffer (pH 7.0) containing 5 mM EDTA and 1 mM DTT. The active fractions of the eluted solutions were concentrated and applied on a gel filtration column (Superdex TM200 26/60, Amersham Biosciences) and separated using 25 mM potassium phosphate buffer (pH 7.0) containing 5 mM EDTA and 1 mM DTT. The collected active fractions were finally purified by ion exchange chromatography (Q Sepharose 26/10, Amersham Biosciences) with a linear gradient of 25 to 200 mM potassium phosphate buffer (pH 7.0) containing 5 mM EDTA and 1 mM DTT.

For Pfαβαβ, the precipitate with ammonium sulfate at 60% saturation was dissolved in 50 ml of 10 mM potassium phosphate buffer (pH 7.0) containing 0.02 mM PLP, 5 mM EDTA, and 1 mM DTT and dialyzed against the same buffer overnight at 4 °C. The dialyzed sample was applied on a column (2.5 × 27 cm) of DEAE-Sephaloc (Amersham Biosciences) with a linear gradient of 25 to 500 mM potassium phosphate buffer (pH 7.0) containing 5 mM EDTA and 1 mM DTT. Next, the collected active fractions were separated by gel filtration (Superdex TM200 26/60, Amersham Biosciences) and finally purified by ion exchange chromatography (Q Sepharose 26/10, Amersham Biosciences) with a linear gradient of 10 to 300 mM potassium phosphate buffer (pH 7.0) containing 5 mM EDTA and 1 mM DTT. PLP at a concentration of 0.1 mM was added to the solutions of the purified Pfβαβ, and Pfαβαβ.

The α subunit from E. coli was purified as already described (31). The β subunit from E. coli was purified as already described (33). All the purified proteins showed a single band on SDS-PAGE.

Protein Concentrations—The protein concentrations were estimated from the absorbance of the protein solution at pH 7.0 using a cell with a light path length of 1 cm. The values of ODI,0.1 cm were 6.92 for Pfα, 10.18 for Pfβαβ subunit, and 9.94 for Pfαβαβ. These values were determined based on protein assay by the Lowry method using bovine serum albumin as the standard protein. The concentrations of Eca, Ecβα, and Ecαβα were determined using ODI,0.1 cm values 4.4 (34), 6.5, and 6.0 (35), respectively.

Ultracentrifugation Analysis—Ultracentrifugation analysis was carried out in a Beckman Optima model XL-A. Sedimentation equilibrium experiments were performed at 20 °C using an An-60 Ti rotor at a speed of 7,000–32,000 × g. Before taking the measurements, the protein solutions were dialyzed overnight against the desired buffer at 4 °C. The experiments at three different protein concentrations between 1.8 and 0.5 mg/ml were run in Beckman 4-sector cells. The partial specific volumes of 0.751 cm3/g for Pfα, 0.743 for Pfβα, and 0.747 for Pfαβαβ were calculated from the amino acid compositions (36). Analysis of the sedimentation equilibria was performed using the program XLAVEL (Beckman, version 2).

Isothermal Titration Calorimetry—Isothermal titration calorimetry (ITC) was performed using an Omega Isothermal Titration Calorimeter (Microcal, Northampton, MA). Prior to the measurements, the solutions of the α and β subunits were dialyzed against 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 0.1 mM DTT, and 0.02 mM PLP. The dialyzed samples were filtered through a 0.22-μm pore size membrane and then degassed in a vacuum. A 10-μl volume of the β subunit at a high concentration was injected into the 1.315-ml sample cell containing the α subunit with a 170-s equilibration period between injections. Integration of the thermogram and the binding isotherm were analyzed using the ITC data analysis module in ORIGIN software (Microcal Software, Northampton, MA).

Differential Scanning Calorimetry—Differential scanning calorimetry (DSC) was carried out using differential scanning microcalorimeters, VP-DSC (Microcal) and Nano-DSC II model 6100 (Calorimetry Science Corp.) at a scan rate of 1 °C/min. Prior to the measurements, the protein solution was dialyzed against buffer described in the legend of Fig. 5. The dialyzed sample was filtered through a 0.22-μm pore size membrane and then degassed in a vacuum. The protein concentrations during the measurements were 0.2–1.4 mg/ml.

RESULTS

Confirmation of Association States of Recombinant βα and αββ from P. furiosus—Pfα, which consists of 248 residues and has a molecular weight of 27,500, is found to exist in a monomer form in solution (29). Ultracentrifugation analysis was used to determine the association forms of the proteins translated by the trpB and trpBA genes from P. furiosus, which were expressed in E. coli. The apparent molecular weights (Mr,app) at various pHs are shown in Fig. 1. The β chain is comprised of 388 residues and the calculated molecular weight is 42,500 (30). The Mr,app of the recombinant αβ was 84,000–88,000 in the pH region above 4.7, indicating that the β chain exists in a dimeric form (Pβα). The Mr,app of the recombinant complex of α with β subunits was almost nearly equal to 2-fold (140,000) the calculated value for αβ around pH 7. These results show that tryptophan synthase from P. furiosus forms a complex of αββ (Pfαβαβ) as observed for prokaryotic tryptophan synthases from mesophiles (24–28) and from the hyperthermophile (37, 38). The Mr,app of the Pfβαβ decreased with decreasing pH below 4.0, resulting in dissociation to a monomer at pH 3.0. As shown in Fig. 1, the Mr,app of Pfαβαβ decreased with decreasing pH between pH 5 and 4, although that of Pfβαβ did not change.

![Fig. 1. pH dependence of apparent molecular weight of Pfβα and Pfαβαβ](image-url)
were recorded as a function of time with successive 10-fold titration for the subunit association monitored by ITC. Heat effects obtained were 1.6 × 10² m⁻¹ for the subunit association at various temperatures are listed in Table I. The stoichiometry (molar ratio of β/α) of association between Pfα and Pfβ was similar to unity and did not depend on temperature. The stoichiometry for EcTSase was 1.5. In a previous study in which Ecα is injected into the Ecβ₂ solution, the stoichiometry is 1.4 (33, 39). The deviation from unity may be due to a decrease in the binding ability of the β subunit with PLP, because both Ecα and Ecβ₂ showed a single band on SDS-PAGE (33). The K values were of the order of 10⁷ m⁻¹ in the temperature region of 40–60 °C for PγTSase and of the order of 10⁶ m⁻¹ in the temperature region of 20–40 °C for EcTSase (Table I and Fig. 3A). The K values of PγTSase were 2 orders higher than those of EcTSase. The negative values of ΔH for the interaction between Pfα and Pfβ were smaller that those in EcTSase (Table I and Fig. 3B). In both cases of PγTSase and EcTSase, the ΔH values linearly correlated with temperature (Fig. 3B). The heat capacity change (ΔCp) obtained from the slope of the linear correlation was estimated to be −1.96 and −5.56 kJ/K per mole of α subunit for PγTSase and EcTSase, respectively. Fig. 4 shows the temperature dependences of ΔG and ΔS together with ΔH. In the case of PγTSase, the summation of small values of −ΔH and −TΔS yielded the Gibbs energy (ΔG) for the subunit binding reaction. In contrast, for EcTSase, the large negative values of ΔH were compensated by using the large values of −TΔS, resulting in a smaller negative ΔG. The subunit association in PγTSase was characterized by a large K, small negative ΔH, small negative ΔCp, and small ΔS in comparison with EcTSase.

### Thermal Stability of Subunits Alone and the Complex—To explore the relationship between the K values and the stability of PγTSase, the thermal stability of each subunit and complex was measured by differential scanning calorimetry (DSC). The DSC measurement was carried out in the alkaline region, because the proteins became turbid by heating at neutral pH and they do not form a complex in the acidic region (Fig. 1). Fig. 3A shows the DSC curves for Pfα, Pfβ₂, and PfαPfβ₂ at pH 9.3–9.4. The Pfα exhibited a DSC curve with a single peak at 87.2 °C (curve a in Fig. 3A). For Pfβ₂, a major peak appeared at 112.2 °C accompanied by a minor broad peak at 94.6 °C (curve b in Fig. 3A). It was confirmed that the major and minor peaks came from the holo-Pfβ₂ and apo-Pfβ₂, respectively. In the case of PfαPfβ₂, separate two peaks appeared at 104.6 and 112.5 °C (curve c in Fig. 3A). The peak on the higher temperature can be assigned to that coming from Pfβ₂ because the peak temperature (112.5 °C) was quite similar to that of Pfβ₂ alone (112.2 °C). Therefore, the peak temperature at the lower temperature could be considered to arise from Pfα. Table II lists the Tₘ values of individual subunits in the isolated and complex forms, where Tₘ values represent the peak temperature of DSC curves. The Tₘ value of Pfα alone was lower by 25 °C than that of Pfβ₂. However, the Tₘ value of Pfα was enhanced by 17.4 °C due to the complex formation. The Tₘ value of Pfβ₂ did not change due to the complex formation. On the other hand, the Tₘ value of Ecα (53.0 °C) slightly increased by 1.7 °C due to the aαβ₂ complex formation at pH 8.4 (curves a and c in Fig. 5B and Table II). The Tₘ value of Ecβ₂ (80.3 °C) at pH 8.2 did not change by complex formation (curves b and c in Fig. 5B and Table II). The stabilization of Pfα due to the complex formation might be correlated with a strong subunit association with a higher K value obtained by ITC. Remeta et al. (41) have reported that the DSC curve of Staαβ₂ at pH 8.0 showed two separate peaks at the denaturation temperatures of isolated Staα and Staβ₂. Each of the Tₘ values of Ecα and Ecβ₂ was similar to those of the reported Staα and Staβ₂, Tₘ values of

**Fig. 2. Typical Isothermal Titration Calorimetry for the Association of an α Subunit with a β₂ Subunit at 40.0 °C and pH 7.0.** The 50 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM DTT, 1.0 mM EDTA, and 0.02 mM PLP was used for the experiments. A, raw data of titration for the subunit association monitored by ITC. Heat effects were recorded as a function of time with successive 10-fold injections of the β₂ subunit into the sample cell containing the α subunit. 1, Pfβ₂ (0.160 mM as a β monomer) was injected into Pfα (0.015 mM); 2, Ecβ₂ (0.174 mM as a β monomer) was injected into Ecα (0.0189 mM); B, integration of the thermogram yielded a binding isotherm that fits a model of one set site (α + β = αβ) by using variables K, n, and ΔH. Each number, 1 and 2, corresponds to that in panel A, respectively. The solid lines represent the nonlinear regression of the data points according to the model. The parameters, association constant (K), stoichiometry (n) (β/α), and enthalpy change (ΔH) upon formation of the αβ₂ complex obtained were 1.6 × 10² m⁻¹, 1.1, and −28.0 kJ/mol of α subunit, respectively, for PγTSase and 3.6 × 10⁵ m⁻¹, 1.4, and −129.0 kJ/mol of α subunit, respectively, for EcTSase.

**Binding Titrations of α with β₂ Subunits by Isothermal Titration Calorimetry—**To examine the inherent feature of the interaction between Pfα and Pfβ₂ in comparison with EcTSase, an isothermal titration calorimetry (ITC) was used in the absence of any substrates or ligands, and the thermodynamic parameters of the binding of Pfα with Pfβ₂ were estimated. The titration in this study was performed by injecting the β₂ subunit into the α subunit, in the calorimetry cell, at various temperatures and pH 7.0, because the solubility of Pfα was not sufficient for making a solution with a high concentration at pH 7.0. This was contrary to the injection used in our previous studies (33, 39). Fig. 2A displays the typical raw data for the calorimetric titration of the α subunit with the β₂ subunit at 40 °C. The binding of Pfα with Pfβ₂ was exothermic. In Fig. 2B the titration curves are plotted as the sum of the heat released by each injection, normalized by the concentration of the α subunit. The ITC titration curves for both PγTSase and EcTSase fitted well to a model of one set site (α + β = αβ) (Fig. 2B) and permitted the extraction of the enthalpy change (ΔH) upon formation of the complex, the association constant (K), and the stoichiometry (n) (40). The Gibbs energy change (ΔG) and the entropy change (ΔS) upon the subunit association can be evaluated using the following equation,

\[ \Delta G = -RT \ln K = \Delta H - T \Delta S \]  

(Eq. 1)

where T and R are the absolute temperature and the gas constant, respectively. The thermodynamic parameters for the subunit association at various temperatures are listed in Table I.

Subunit Interaction and Stability of Hyperthermophile Tryptophan Synthase
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Thermodynamic parameters of the association of the α subunit with the β subunit in PfTSase, EcTSase, and hybrid complexes between subunits from PfTSase and EcTSase obtained by ITC measurements at pH 7.0.

Parameters obtained by ITC are represented per molar concentration of α subunit.

| Temperature (°C) | n (μmol) | K | ΔH | ΔG | ΔS | ΔCp |
|-----------------|---------|---|----|----|----|-----|
| **Pfαβ association** | | | | | | |
| 35              | 1.2     | 0.7 | -9.4 | -46.3 | 119.6 | -1.96 |
| 40              | 1.1     | 1.6 | -26.0 | -49.2 | 74.0  |      |
| 43.1            | 1.2     | 2.7 | -27.2 | -51.0 | 75.4  |      |
| 45              | 1.2     | 3.1 | -33.2 | -51.7 | 58.3  |      |
| 47              | 1.1     | 3.9 | -38.4 | -52.6 | 44.5  |      |
| 50              | 1.1     | 4.0 | -43.9 | -53.2 | 28.0  |      |
| 55              | 1.1     | 6.5 | -53.4 | -55.4 | 5.9   |      |
| 60              | 1.2     | 9.5 | -58.7 | -57.3 | 4.3   |      |
| **Ecαβ association** | | | | | | |
| 21              | 1.5     | 0.0293 | -15.4 | -36.4 | 71.7  | -5.56 |
| 25              | 1.5     | 0.0286 | -33.5 | -36.9 | 11.4  |      |
| 30              | 1.5     | 0.0599 | -56.1 | -39.3 | -55.5 |      |
| 35              | 1.5     | 0.0400 | -87.4 | -38.9 | -157.1 |      |
| 37              | 1.5     | 0.0422 | -101.4 | -39.4 | -200.0 |      |
| 37              | 1.4     | 0.0340 | -104.4 | -38.8 | -211.5 |      |
| 38              | 1.5     | 0.0322 | -105.4 | -38.8 | -213.9 |      |
| 40.07           | 1.4     | 0.0356 | -129.0 | -39.3 | -286.3 |      |
| **Hybrid α/β association** | | | | | | |
| PfαEcβ2 40      | 1.1     | 0.00470 | -24.0 | -34.0 | 32.0  |      |
| EcαPfβ2 40      | 0.7     | 0.00061 | -2.9  | -28.7 | 82.0  |      |

**Fig. 3.** Temperature dependences of the association constant and enthalpy change upon association of the α subunit with the β subunit at pH 7.0. A and B display the temperature dependence of K and ΔH, respectively. Closed and open circles denote the values for PfTSase and EcTSase, respectively. In panel B, the linear lines show the linear correlation between ΔH and temperature. ΔCp values obtained from the slope of a line were ~1.96 and ~5.56 kJ/K mol of the α subunit for PfTSase and EcTSase, respectively.

**Fig. 4.** Temperature dependences of enthalpy change, entropy change, and Gibbs energy change upon association of the α with β subunits at pH 7.0. A and B display the temperature dependence for PfTSase and EcTSase, respectively. Open triangles, ΔH; open circles, -TΔS; closed circles, ΔG.

Pfα and Pfβ2 were drastically higher by 34.2 and 31.9 °C than those of Ecα and Ecβ2, respectively.

**ITC and DSC Measurements of Hybrid Complex Between Pf Subunits and Ec Subunits**—To explore which of the α and (or) β subunits corresponds to the strong association in PfTSase, the interaction between the Pf subunits and Ec subunits was examined by ITC. The ITC data at 40 °C demonstrated that the K values upon formation of the hetero complex between the Pf subunits and Ec subunits were lower than those of the homo complexes (Table I). The K value strongly decreased by 4 and 3 orders of magnitude for the Pfβ2-Ecα and Ecβ2-Pfα associations, respectively, compared with that for the Pfα-Pfβ2 association. These results suggest that the conformation of the subunit interface in PfTSase differs from that in EcTSase.

**DISCUSSION**

**The Conformational Change upon the Subunit Association of PfTSase**—The K values of PfTSase in the range from 40 to 60 °C were of the order of 10^3 M^-1 and 2 orders higher than those of EcTSase in the range from 20 to 40 °C (Table I and Fig. 3A). This means that the interaction between the α and β
subunits from the hyperthermophile is extremely strong. The protein-protein association with a K value of 8 orders or over has been reported in the hen egg white lysozyme, its antibody (42–45), barnase-barstar (46), and transthyretin-retinol binding protein (47) interactions, which are highly specific for biological significance. The association between the subunits in P/Tsase is equivalent to such a highly specific interaction.

The negative of ∆Cp of the subunit association for P/Tsase (~1.96 kJ/K mol of α) was less than half of that for EcTsase (−5.56 kJ/K mol of α) (Table I and Fig. 3B). In the cases of Tsases from mesophiles, Hiraga and Yutani (39) have reported that a heat capacity change (ΔCp_est) is estimated to be −1.05 kJ/K mol from the values of the water-accessible nonpolar (∆Aα np) and polar (∆Aα p) surface areas buried upon subunit association in the α/β subunit interface in the crystal structure of the StTsase complex. The negative values of ΔCp experimentally obtained upon the subunit association for EcTsase and StTsase (~7.29 and −6.83 kJ/K mol, respectively) are much larger than the estimated one (~1.05 kJ/mol) mentioned above.

It has been evaluated that this difference comes from the folding of many residues coupled to the α/β subunit association in EcTsase and StTsase (39), according to the method of Spolar and Record (48). ∆Aα np and ∆Aα p in the α/β interface for P/Tsase complex can not be estimated, because the structure of the P/Tsase complex has not yet been determined. However, the number of residues of local folding coupled to the subunit association in P/Tsase might be postulated to be slight, because of the smaller negative ∆Cp (~1.96 kJ/K mol of α). For a rigid body association in which a specific site is recognized by a “lock and key” interaction, an experimental ΔCp value might be similar to the ΔCp_est predicted from ∆Aα np and ∆Aα p, resulting from burial of the pre-existing complementary surface (48). Negative ΔCp values of association corresponding to the concept of “induced fit” are larger than the negative ΔCp_est predicted from ∆Aα np and ∆Aα p. In this case, the folding of many residues is coupled to the association and creates key parts of the protein-protein, protein-ligand, and protein-DNA interface (48). According to this criteria, the subunit association in P/Tsase resembles a rigid body association. In contrast, the subunit association in EcTsase corresponds to an “induced fit” with large conformational changes.

The smaller negative ΔH of the subunit association for P/Tsase relative to that for EcTsase (Table I and Fig. 3B) also indicates that the conformational changes upon the subunit association are much smaller in P/Tsase than in EcTsase, because the negative value of the enthalpy change due to protein folding is high (49). In P/Tsase, the small values of negative ΔH and positive ΔS yield the negative value of ΔG for driving the subunit association (Fig. 4A). The association constant between the hetero subunits from P/Tsase and EcTsase at 40 °C were drastically decreased relative to those for P/Tsase and EcTsase, resulting in the decreases in the negative ΔG (Table I). The negative ΔH for the hetero subunit associations also decreased. These results indicate that the α and β subunits of P/Tsase cannot strongly bind to each of subunits from EcTsase. The thermodynamic parameters of the subunit associations revealed that the binding between the α and β subunits was much tighter in P/Tsase than in EcTsase, and the conformational change coupled with the subunit association was low in P/Tsase.

**Structural Bases of Strong Subunit Association in P/Tsase**—
The structures of the Stαβ2 complex form (23, 50–52) and an isolated Pfα monomer (29) have been determined by x-ray analysis. We tried to explore the cause responsible for the strong subunit association in P/Tsase from comparison of the structures of the subunit interfaces in Pfα and Tsα. The crystal structure of Pfα alone (29) is the same topological pattern to that of Stα in the Stαβ2 complex form (23). Pfα, Stα, and Ecα consist of 248, 268, and 268 residues, respectively. The sequence identities between Pfα and Stα and between Ecα and Stα are 31.5 and 85.1%, respectively.

We can find out the differences in the two structures as follows. The loops 2 and 6 in the Stα, which play an important role in the catalysis and allosteric communication between the active sites of the α and β subunits, contact with Stβ (23, 50–52). The B-factor averaged for the main-chain atoms of the loop 2 in Pfα is considerably lower than that in Stα (29), indicating that the loop 2 is less mobile in Pfα than in Stα. The loop 6 of Stα is highly mobile and 12 residues in the loop 6 have not been determined due to a weak electron density (23). In Pfα, only three residues are not determined, although Pfα does not form a complex with the β subunit, indicating that the number of mobile residues in Pfα is drastically reduced (29). The amino acid residues of the loop 6 in Pfα exchange by polar to nonpolar, acidic to basic, or less to more hydrophobic resi-
are the residues in the loop 2 is highly conserved in both St and Pf. However, remarkable deviations in the root mean square deviations of the residues of Val119, Phe120, and His121 in St are found in the C-terminal subunits (Fig. 6). The differences in the $T_d$ values of individual subunits in the isolated and homo complex forms.

### TABLE II

| Subunits | $T_d$ (°C) Isolated form | $T_d$ (°C) Homo complex form | $T_d$ (°C) Hybrid complex form | $\Delta T_d$ (°C) |
|----------|--------------------------|-----------------------------|-------------------------------|-----------------|
| Pfα      | 87.2 (pH 9.37)           | 104.6 (pH 9.30)             | 86.4 (pH 9.50)                | 17.4            |
| Pfβ      | 112.2 (pH 9.30)          | 112.5 (pH 9.30)             | 111.6 (pH 9.49)               | 0.3             |
| Ecα      | 53.0 (pH 8.40)           | 54.7 (pH 8.40)              | 53.5 (pH 9.49)                | 1.7             |
| Ecβ      | 80.3 (pH 8.21)           | 79.9 (pH 8.40)              | 71.5 (pH 9.50)                | −0.4            |

The $T_d$ values represent the peak temperature of the DSC profiles measured at scan rate of 1 °C/min.

1. From panels A and B in Fig. 5.
2. From panel C in Fig. 5 for the hybrid complexes between the Pf subunits and Ec subunits.
3. The differences in the $T_d$ values of individual subunits in the isolated and homo complex forms.

### FIG. 8

**The amino acid sequences for Pfα, Ecα, and Sta in the interfaces of the α and β subunits.** Bold show residues that differed from Stα. Residues numbers of Pfα and Stα are shown.

| Residue No. | Loop2 | Hydrogen bonds in St | Loop6 |
|-------------|-------|----------------------|-------|
| Pf          | IPFSADLADG | VULPVHAKP | VSLYSTGAREK |
| Ec          | IPFSADLADG | ADVVFAAPP | LSRGTVGARENAL |
| St          | IPFSADLADG | ADVVFAAPP | LSRGTVGARENAL |

### TABLE III

The estimations of increment in denaturation temperature ($\Delta T$) of Pfα due to $K_{βα}$ complex formation using the association constant (K) at different temperatures

$\Delta n$ is the molar ratio of the association (Pfβ per Pfα). $\Delta T$ and $\Delta n$ were evaluated according to Equation 2. In Equation 2, $T_0$ and $T$ are the denaturation temperatures of Pfα alone (360.35 K) and of Pfα in Pfαβ complex form, respectively (Fig. 5). $L$ is the concentration (8.72 × 10−6 M) of monomer Pfβ under DSC measurements.

| Temperature (°C) | 40.0 | 43.1 | 45.0 | 47.0 | 50.0 | 55.0 | 60.0 |
|------------------|------|------|------|------|------|------|------|
| $\Delta T$ (°C)  | 15.6 | 16.8 | 17.1 | 17.6 | 17.6 | 18.7 | 19.5 |
| $\Delta n$ (βα)  | 0.9  | 0.9  | 0.9  | 0.9  | 0.9  | 0.9  | 0.9  |

* Temperature under ITD measurements (Table I). The K used in Equation 2 was obtained by ITC measurements at various temperatures listed in Table I. $\Delta T$ was calculated assuming $\Delta n$ of 1.0. $\Delta n$ was calculated using 17.4 °C as $\Delta T (= T_0 − T_d)$.

### Dues from those in STTαase, although the amino acid sequence of the loop 2 is highly conserved in both α subunits (Fig. 6). In other regions of the subunit interface in STTαase, six hydrogen bonds are formed (53). The corresponding residues in PFTTαase are presented in Fig. 6. The hydrogen bonding residues in STTαase are not conserved in both the α and β subunits in PFTTαase except for the Asn104-Gly129 pair in STTαase. The remarkable deviations in the root mean square deviations of the Ca atoms between Pfα and Sta are found in the loop 2 and in the residues of Val119, Phe120, and His121 in Pfα (29), which are the residues in Sta forming hydrogen bonds with the residues in Stβ. From these observations, it seems that the conformations of the interface in Pfα substantially differ from those in Sta, and the rigidly ordered conformations of the loops 2 and 6 in Pfα might contribute to creating the key part of the interface responsible for the strong subunit association in PFTTαase.

**Correlation between Subunit Association Constant and Protein Stability**—For dimeric phosphoribosylanthranilate isomerase (7) and tetrameric pyrrolidine carboxyl peptides (54) from hyperthermophiles and dimeric 3-isopropylmalate dehydrogenase from thermophiles (55), the experimental data have shown that the subunit interaction is important to the increase in thermal stability in solution. For these proteins, however, the relationship between subunit association and stability has not been quantitatively evaluated. Schellman (56, 57) has developed an equation for the relationship between the binding constant of a ligand to a biopolymer and the melting temperature of a biopolymer as follows,
M$^{-1}$. Because the conformational change coupled to the subunit association in PT$\beta$ase was low as judged from the thermodynamic parameters for the subunit association, it was proved that the increase in $T_d$ of $Pf\beta$ due to the complex formation is not due to the subunit binding-induced conformational stabilization of the subunits but due to the shift in the equilibrium toward the native state, which is caused by the increase in the association constant. This reveals that the enhancement in the thermal stability of subunit resulting from subunit association can be quantitatively evaluated by the subunit association constant.

The $T_d$ values of the $Pf\beta$ monomer in the acidic region remarkably decreased with lower pH levels,2 suggesting the importance of the $\beta$-$\beta$ subunit interaction for the higher stability of $Pf\beta_2$ compared with $Pf\alpha$. The $T_d$ (80.3 °C) value of $Eca\beta_2$ at pH 8.4 was higher than that of $Eca$ and comparable to that of $Pf\alpha$ at pH 9.4 (Fig. 5, A and B). The interaction between the $\beta$ subunits from the mesophile also enhances the stability of this dimeric protein.

Conclusion—The present study proved four significant aspects of the subunit association in the PT$\beta$ase compared with Ec$\beta$ase: 1) $Pf\alpha$ and $Pf\beta_2$ tightly bind by the $K$ value of the order of $10^3$ M$^{-1}$; 2) The negative values of $\Delta C_p$ and $\Delta H$ on the subunit association were low, indicating that the conformational change coupled to the subunit association is low; 3) The $T_d$ of $Pf\alpha$ was drastically enhanced by 17 °C in the $\alpha_2\beta_2$ complex form; 4) This increment could be quantitatively evaluated from the remarkably increased $K$ value. It was found that the stimulated interaction between the subunits with the order of $10^8$ M$^{-1}$ or over of $K$ values remarkably enhances the thermal stability of a protein without conformational changes.

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2 K. Ogasahara, M. Ishida, and K. Yutani, unpublished data.
Additions and Corrections

Vol. 278 (2003) 11818–11827

The unfolded protein response is required for haploid tolerance in yeast.

Kyungho Lee, Lenore Neigeborn, and Randal J. Kaufman

Page 11818: The symbol for Dr. Neigeborn’s affiliation was incorrect. The corrected author list with affiliations is shown below:

Kyungho Lee‡§, Lenore Neigeborn¶, and Randal J. Kaufman‡

From the ‡Howard Hughes Medical Institute, Department of Biological Chemistry, University of Michigan Medical Center, Ann Arbor, Michigan 48109 and ¶Rutgers College, New Brunswick, New Jersey 08901

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Stimulated interaction between α and β subunits of tryptophan synthase from hyperthermophile enhances its thermal stability.

Kyoko Ogasahara, Masami Ishida, and Katsuhide Yutani

Page 8924, Fig. 2B: The line below the figure should read molar ratio of β/α. The denominator and the numerator were mistakenly reversed. The corrected figure is shown below.

![Corrected Figure 2B](image)

We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.