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Subunit Interaction in Tryptophan Synthase of *Escherichia coli*: Calorimetric Studies on Association of α and β_2 Subunits†

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ABSTRACT: Association of the apo-β_2 and the holo-$(\beta$-PLP$)_2$ subunits of tryptophan synthase from *Escherichia coli* (L-serine hydro-lyase (adding indole) (EC 4.2.1.20)) with α subunits of the same enzyme has been studied by microcalorimetry. The results obtained from thermometric titrations clearly demonstrate that only the native complex $\alpha_2\beta_2$ is formed, independent of an excess of α protein. The reaction of the holo-$(\beta$-PLP$)_2$ with α subunits at 25 °C is accompanied by a negative enthalpy change, which is almost twice as large as that for complex formation with the apo-β_2 protein, thus indicating that the interaction enthalpy becomes more favorable in the presence of the coenzyme pyridoxal 5′-phosphate (PLP). Both reaction enthalpies show very large negative temperature coefficients, -3600 ± 100 cal K^{-1} (mol of $\beta_2)^{-1}$ being the value for the formation of the apoenzyme and -2300 ± 100 cal K^{-1} (mol of $\beta_2)^{-1}$ pertaining to formation of the holoenzyme. The studies on the association of α and β_2 subunits in the two buffers revealed that at 25 °C approximately 0.75 proton are absorbed in the presence and absence of the coenzyme, whereas at 35 °C one proton is taken up from the solution when PLP is present, but two if the apo-β_2 complex reacts. These results are a clear indication of energetic linkage between intersubunit interaction, hydrogen ion equilibria, and the binding of the coenzyme.

The interaction of subunits in multimeric enzymes constitutes an informative example of protein–protein interaction. The energetics operative in maintenance of the quaternary structure and the influence exerted by small ligands on the thermodynamic interaction parameters can be particularly well illustrated, if reaction enthalpies and their temperature coefficients are available in addition to the equilibrium constants. Although generalization is not too safe due to the scarcity of relevant data, reaction enthalpies appear to exhibit often larger absolute changes in magnitude than Gibbs free energies, thus rendering them appropriate quantities for the characterization of the energetic aspects of the reaction. The particular reaction chosen is the reconstitution of tryptophan synthase (L-serine hydro-lyase (adding indole) (EC 4.2.1.20)) from the β_2 subunits and α subunits in the presence and absence of the coenzyme pyridoxal 5′-phosphate (PLP[1]). Evidence has been presented on the basis of kinetic and binding studies (Faeder & Hammes, 1971; Weischet & Kirschner, 1976; Kirschner et al., 1975a,b; Heyn & Weischet, 1975; Kirschner & Wiscocil, 1972; Bartholmes et al., 1976) performed with the isolated subunits β_2 and α, as well as with the native tetrameric enzyme

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[1] Abbreviations used: DTE, dithioerythritol; PLP, pyridoxal 5′-phosphate.

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$\alpha_2\beta_2$, that heterologous interaction between α and β_2 subunits is responsible for the observed simultaneous increases in catalytic efficiency and affinity for substrates. The modifications of the protein structure associated with these effects are a result of the inevitable linkage between internal protein interactions and protein–ligand interactions. Thus the determination of reaction enthalpies and their temperature coefficients for the reconstitution of tryptophan synthase had two aims: first, to make the energetics involved in keeping up the quaternary structure of the enzyme more understandable and, second, to provide some thermodynamic parameters pertinent to protein–protein interaction.

Materials and Methods

Materials

Enzymes. The α subunit of tryptophan synthase was prepared following the procedure published by Kirschner et al. (1975a) and stored as an ammonium sulfate suspension at −4 °C. The β_2 subunit was prepared as described by Bartholmes et al. (1976) and kept at −75 °C in 0.6 M potassium phosphate buffer, pH 7.8, 1 mM DTE, 5 mM EDTA, 2 mM PLP, until use. Activity and concentration determinations were performed as described in these two references. The reassociated enzyme was routinely checked for activity and was found to display the same activity as the native species (3200–4000 Yanofsky units).

Buffers and Chemicals. Unless stated otherwise, all experiments were performed in 0.1 M sodium pyrophosphate buffer adjusted with HCl to pH 7.5 or in 0.2 M Tris-HCl buffer, pH 7.5, adjusted with sodium chloride to the same ionic strength as the phosphate buffer. For measurements in the presence of the coenzyme, the buffer solutions contained 0.02 mM PLP. When Tris buffer was used, the PLP concentration was 0.08 mM. PLP was purchased from Serva, Heidelberg, and DTE from Roth, Karlsruhe. All other chemicals were obtained from Merck, Darmstadt, and were of reagent grade quality.

Methods

Absorption measurements were performed at 25 ± 0.2 °C using a Varian Cary 118 spectrophotometer. The calorimetric measurements were performed using a recently developed flow microcalorimeter (Weber & Hinz, 1976). The instrument was operated in the pulsed flow mode, with pulse duration between 20 and 50 s. During a period of 50 s, 0.2999 mL each of solutions of α and β_2 were mixed in the calorimeter, the concentration of the subunits before mixing ranging from approximately 4 to 10 mg/mL. For the determination of the saturation enthalpies, the α subunits were present in excess. All molar values of ΔH have been referred to the molecular weight of 89 000 for the β_2 subunit. They have been corrected for dilution effects of the components; these corrections never exceeded 3%.

Preparation of Enzyme Solutions. Prior to the calorimetric measurements, 2–3-mL portions of the concentrated stock solutions of the α and β_2 subunits (30–40 mg/mL) were dialyzed for a total of 24 h against 1 L of the buffer solution to be employed in the experiments, with three changes of the buffer.

Occasionally occurring turbidity of the dialyzed solution, indicating formation of higher aggregates, was eliminated by Millipore filtration (45-μm pore size). The enzyme concentrations used in the calorimetric studies were adjusted with the dialysate. pH values of the solutions were measured at the temperature of the experiments using a digital pH meter (WTW, Weilheim).

Table I: Enthalpies of Association of α and Apo-β_2 Subunits of Tryptophan Synthase at 25 °C in 0.1 M Pyrophosphate Buffer, pH 7.5, in the Presence of an Excess of α Subunits

$[\alpha]^a$ (mM)	$[\beta_2]^a$ (mM)	molar ratio α/β	N^b	ΔH (kcal/mol of β_2)
0.107	0.030	1.8:1	5	−13.7 ± 1.1[c]
0.325	0.075	2.2:1	7	−14.6 ± 0.5
0.368	0.073	2.5:1	8	−10.6 ± 1.6
0.405	0.087	2.3:1	7	−10.9 ± 1.5
0.209	0.030	3.5:1	4	−14.1 ± 1.8
0.640	0.030	10.7:1	4	−14.4 ± 2.2

[a] Concentrations of α and β_2 subunits, respectively, before mixing in the calorimeter. [b] Number of measurements. [c] Standard error of the measurements.

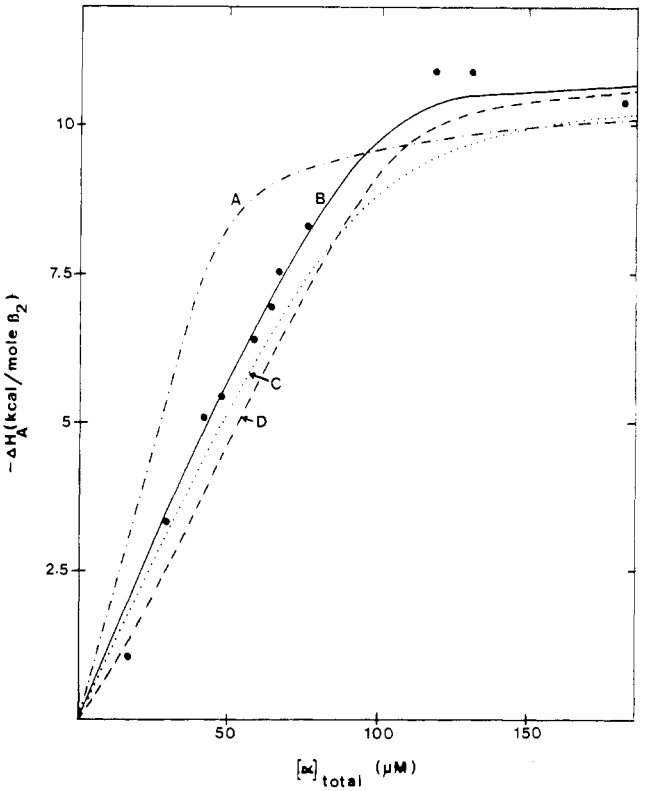

FIGURE 1: Enthalpimetric titration of β_2 subunits of tryptophan synthase with α subunits. $[\beta_2]$ = 94 μM binding sites after mixing; sodium pyrophosphate buffer, 0.1 M, pH 7.5. The points refer to the experimental values. Curve B obviously provides the best fit. It has been calculated on the basis of the following parameters: K_{D1} = 28 μM, K_{D2} = 0.8 μM; ΔH_1 = 9.0 kcal/mol of β_2, ΔH_2 = 1.71 kcal/mol of β_2. To demonstrate the quality of the fit, curves A, C, and D have been generated using the following parameters: (A) K_{D1} = 0.8 μM, K_{D2} = 28 μM; ΔH_1 = 9.0 kcal/mol of β_2, ΔH_2 = 1.71 kcal/mol of β_2; (C) identical sites; $K_{D1} = K_{D2} = (K_{D1}K_{D2})^{1/2}$ = 4.73 μM; $\Delta H_1 = \Delta H_2$ = 5.355 kcal/mol of β_2; (D) K_{D1} = 28 μM, K_{D2} = 0.8 μM; ΔH_1 = 1.71 kcal/mol of β_2, ΔH_2 = 9.0 kcal/mol of β_2.

Results

Stoichiometry of Complex Formation. To check whether higher aggregates than the $\alpha_2\beta_2$ complex are formed on reassociation of tryptophan synthase from β_2 and α subunits, an enthalpimetric titration in the absence of the coenzyme as well as various determinations of the overall saturation enthalpy employing different molar ratios of α:β_2 have been performed in the presence and absence of PLP with the results shown in Tables I and II and Figure 1. The experimental values in Figure 1 can be interpreted without a detailed mathematical analysis to reflect rather tight binding since the heat released on complex formation increases almost linearly within experimental error with increasing concentration of α

Table II: Enthalpies of Association of α and Holo-(β-PLP)$_2$ Subunits of Tryptophan Synthase at 25 °C in 0.1 M Pyrophosphate Buffer, pH 7.5, Containing 0.02 mM PLP, in the Presence of an Excess of α Subunits

$[\alpha]^a$ (mM)	$[\beta_2]^a$ (mM)	molar ratio α/β	N^b	ΔH (kcal/mol of β_2)
0.242	0.092	1.3:1	6	−18.7 ± 1.5[c]
0.349	0.060	2.9:1	6	−21.5 ± 0.5
0.240	0.040	3.0:1	7	−21.7 ± 1.7
0.340	0.082	2.1:1	6	−22.8 ± 0.4
0.349	0.060	2.9:1	5	−21.5 ± 0.5
0.336	0.060	2.8:1	7	−25.0 ± 1.8
0.340	0.067	2.5:1	6	−22.5 ± 1.3

[a] Concentrations of α and β_2 subunits, respectively, before mixing in the calorimeter. [b] Number of measurements. [c] Standard error of the measurements.

subunits. There is a rather abrupt change to a concentration independent saturation value at an α concentration which corresponds very closely to a 2:1 ratio of α:β_2 subunits, thus suggesting the existence of uniquely formed $\alpha_2\beta_2$ species under the experimental conditions employed in the calorimeter. This result is corroborated by the fact that the saturation enthalpies, given in Tables I and II, are independent of the excess of α subunits both for the apoenzyme and the holoenzyme. Therefore one can safely conclude that the observed heat effects are due to formation of the stoichiometric $\alpha_2\beta_2$ tryptophan synthase complex.

Curve B in Figure 1 has been calculated on the basis of the two dissociation constants, K_{D1} = (28 ± 3) μM and K_{D2} = (0.8 ± 0.1) μM, for the two binding sites of the β_2 subunit (Bartholmes & Teuscher, 1979) and the enthalpies associated with complex formation of α subunits with sites 1 and 2, respectively, ΔH_1 = 9.0 kcal/mol of β_2 and ΔH_2 = 1.71 kcal/mol of β_2. The quality of the fit decreases if one assumes a smaller ΔH_1 value than 8.5 kcal/mol of β_2 or a larger than 9.5 kcal/mol of β_2. Thus at 25 °C the larger dissociation constant of the first binding site (28 μM) is accompanied by the larger enthalpy.

Temperature Dependence of the Reaction Enthalpies. Characterization of the energetics of reactions involving biological macromolecules is incomplete unless the variation with temperature of the reaction enthalpies has been determined, since large heat capacity changes appear to be the rule rather than the exception (Hinz et al., 1971, 1976, 1977a; Bode et al., 1974; Johnston et al., 1974; Hinz & Jaenicke, 1975; Schmid et al., 1976; Kodama & Woledge, 1976; Hinz & Schmid, 1977; for a review, see Sturtevant, 1977). Therefore, reaction enthalpies of $\alpha_2\beta_2$ complex formation were also determined at 10 and 35 °C. The results of these experiments are compiled in Tables III and IV. Table III shows the saturation enthalpies of the reaction at 10 °C both for the apo- and holoenzyme, while Table IV presents the corresponding quantities for 35 °C.

A prominent feature of these results is the pronounced temperature dependence of the ΔH values for the recombination of both the apo- and the holoenzyme. A linear regression analysis employing the ΔH values at 10, 25, and 35 °C results in eq 1 and 2, in which ϑ is the temperature in °C and the enthalpy is expressed in kcal per mol of β_2. One

apoenzyme: ΔH_A =
$$[-(3.6 \pm 0.1)\vartheta + 77.6] \text{ kcal/mol of } \beta_2 \quad (1)$$

holoenzyme: ΔH_A =
$$[-(2.3 \pm 0.1)\vartheta + 36.0] \text{ kcal/mol of } \beta_2 \quad (2)$$

obtains a somewhat better fit of the experimental data when a regression analysis of the second degree is performed. This would imply a temperature-dependent ΔC_p. However, within the margin of experimental error such a refinement does not appear to be justified.

Table III: Enthalpies of Association of α and β_2 Subunits of Tryptophan Synthase at 10 °C in 0.1 M Pyrophosphate Buffer, pH 7.5, in the Presence of Saturating Concentrations of α Subunits[a]

enzyme	$[\alpha]^b$ (mM)	$[\beta_2]^b$ (mM)	molar ratio α/β	T (°C)	N^c	ΔH (kcal/mol of β_2)
apo	0.463	0.081	2.9:1	10	4	43.5 ± 3.8[d]
	0.288	0.092	1.5:1	10	9	47.4 ± 2.6
	0.341	0.081	2.1:1	10	7	38.8 ± 1.3
holo	0.145	0.038	1.9:1	10	5	11.3 ± 0.3
	0.279	0.082	1.7:1	10	5	14.1 ± 1.7
	0.279	0.053	2.6:1	10	9	14.6 ± 0.8

[a] Reactions of α and holo-(β-PLP)$_2$ proteins were performed in the same buffer containing 0.02 mM PLP. [b] Concentrations of α and β_2 subunits, respectively, before mixing in the calorimeter. [c] Number of measurements. [d] Standard error of the measurements.

Table IV: Enthalpies of Association of α and β_2 Subunits of Tryptophan Synthase at 35 °C in 0.1 M Pyrophosphate Buffer, pH 7.5, in the Presence of Saturating Concentrations of α Subunits[a]

enzyme	$[\alpha]^b$ (mM)	$[\beta_2]^b$ (mM)	molar ratio α/β	T (°C)	N^c	ΔH (kcal/mol of β_2)
apo	0.289	0.057	2.5:1	35	6	−43.9 ± 1.8[d]
	0.280	0.053	2.6:1	35	7	−44.8 ± 2.2
holo	0.310	0.067	2.3:1	35	7	−42.8 ± 1.6
	0.300	0.055	2.7:1	35	7	−44.9 ± 2.5

[a] Reactions of α and holo-(β-PLP)$_2$ proteins were performed in the same buffer containing 0.02 mM PLP. [b] Concentrations of α and β_2 subunits, respectively, before mixing in the calorimeter. [c] Number of measurements. [d] Standard error of the measurements.

Involvement of Proton Equilibria in Subunit Interaction. A contribution to stability of the quaternary structure of tryptophan synthase, which can be singled out rather elegantly by calorimetric studies, is the participation of hydrogen ions in the binding equilibria between the subunits. For this to be accomplished, one determines the reaction enthalpies in a second buffer of different heat of ionization (Beres & Sturtevant, 1971; Hinz et al., 1971). As an appropriate buffer, Tris has been employed, since it has a high heat of ionization (Grenthe et al., 1970) and is generally used in the activity test of tryptophan synthase. The results of these studies are summarized in Tables V and VI, which show the saturation enthalpies for formation of the apoenzyme and the holoenzyme, respectively, at 25 and 35 °C in 0.2 M Tris-HCl buffer, pH 7.5.

Table VII provides a survey of the average ΔH values, $\overline{\Delta H_A}$, of all determinations in phosphate and Tris buffers as well as the difference $\Delta\overline{\Delta H_A}$ between the enthalpies obtained in the two buffer systems. These differences document a very surprising variance between the reactions of the apo- and holoenzyme. Whereas at 25 °C complex formation of α and β_2 subunits apparently is associated with an absorption of approximately 0.75 proton per β_2 subunit both with and without PLP present, the stoichiometry is changed at 35 °C. At this temperature, association of the subunits in the absence of the coenzyme is linked to absorption of two protons per β_2 subunit within experimental error, since the heat of ionization of Tris at 35 °C is −11.18 kcal/mol (Grenthe et al., 1970) and that of pyrophosphate buffer +0.75 kcal/mol (Christensen et

Table V: Enthalpies of Association of α and β_2 Subunits of Tryptophan Synthase at 25 °C in 0.2 M Tris-HCl Buffer, pH 7.5[a]

enzyme	$[\alpha]^b$ (mM)	$[\beta_2]^b$ (mM)	molar ratio α/β	T (°C)	N^c	ΔH (kcal/mol of β_2)
apo (Tris buffer)	0.352	0.062	2.8:1	25	6	−4.4 ± 0.3[d]
	0.372	0.077	2.4:1	25	7	−5.2 ± 0.3
	0.370	0.067	2.7:1	25	7	−4.3 ± 0.3
holo (Tris buffer)	0.348	0.077	2.2:1	25	9	−14.9 ± 1.1
	0.290	0.069	2.1:1	25	7	−12.7 ± 1.3
	0.203	0.054	1.8:1	25	7	−13.0 ± 0.4
	0.216	0.059	1.8:1	25	7	−14.4 ± 1.6

[a] The ionic strength was adjusted to that of the pyrophosphate buffer by addition of sodium chloride. Reactions of α and holo-(β-PLP)$_2$ proteins were performed in the same buffer containing 0.08 mM PLP. [b] Concentrations of α and β_2 subunits, respectively, before mixing in the calorimeter. [c] Number of measurements. [d] Standard error of the measurements.

Table VI: Enthalpies of Association of α and β_2 Subunits of Tryptophan Synthase at 35 °C in 0.2 M Tris-HCl buffer, pH 7.5[a]

enzyme	$[\alpha]^b$ (mM)	$[\beta_2]^b$ (mM)	molar ratio α/β	T (°C)	N^c	ΔH (kcal/mol of β_2)
apo (Tris buffer)	0.354	0.047	3.8:1	35	9	−22.7 ± 1.6[d]
	0.333	0.051	3.2:1	35	7	−22.9 ± 1.5
holo (Tris buffer)	0.257	0.067	1.9:1	35	8	−29.4 ± 1.4
	0.274	0.061	2.2:1	35	4	−32.9 ± 1.1

[a] The ionic strength was adjusted to that of the pyrophosphate buffer by addition of sodium chloride. Reactions of α and holo-(β-PLP)$_2$ proteins were performed in the same buffer containing 0.08 mM PLP. [b] Concentrations of α and β_2 subunits, respectively, before mixing in the calorimeter. [c] Number of measurements. [d] Standard error of measurements.

Table VII: Average Enthalpies of Association, $\overline{\Delta H}$, of α and β_2 Subunits of Tryptophan Synthase in Pyrophosphate and Tris Buffers at Various Temperatures in the Presence and Absence of PLP

buffer	enzyme	T (°C)	$\overline{\Delta H}$ (kcal/mol of β_2)	$\Delta\overline{\Delta H}^a$ (kcal/mol of β_2)
0.2 M Tris-HCl,[b,c] pH 7.5	holo	25	−13.7 ± 1.0[d]	8.3 ± 1.0
		35	−31.1 ± 1.4	12.3 ± 1.4
	apo	25	−4.6 ± 0.5	8.5 ± 0.5
		35	−22.8 ± 1.6	21.6 ± 1.6
0.1 M $NaPP_i$,[e] pH 7.5	holo	10	+13.3 ± 1.8	
		25	−22.0 ± 1.8	
		35	−43.4 ± 1.9	
	apo	10	+43.2 ± 4.3	
		25	−13.1 ± 1.8	
		35	−44.4 ± 2.0	

[a] $\Delta\overline{\Delta H} = \overline{\Delta H}$ (in Tris buffer) − $\overline{\Delta H}$ (in pyrophosphate buffer). [b] Ionic strength adjusted to that of the pyrophosphate buffer by addition of sodium chloride. [c] For measurement on holoenzyme formation the buffer contained 0.08 mM PLP. [d] Standard error of all measurements under identical conditions are taken into account. [e] For measurement on holoenzyme formation the buffer contained 0.02 mM PLP.

al., 1976). However, formation of the holoenzyme, i.e., binding of α subunits to β_2 subunits in the presence of saturating conditions of PLP, evidently is accompanied by an uptake of one proton per β_2 subunit.

Thus on the basis of the calorimetric results, the equation describing the overall equilibrium between α and β_2 subunits must be formulated:

$$2\alpha + \beta_2' + n\mathrm{H}^+ = \alpha_2\beta_2'\mathrm{H}^+_n$$

with $n = 0.75$ for the holo- and apoenzyme reassociation at 25 °C and $n = 1$ and 2, respectively, at 35 °C. β_2' denotes the β_2 subunit both in the presence and the absence of PLP.

Discussion

Stoichiometry of the Complexes. Although tryptophan synthase can be considered as belonging to the class of rather well-characterized enzymes (see the review by Yanofsky & Crawford, 1972), there is no information on the energetics other than the standard free energies of its various reactions. With respect to some binding constants, there does not exist good agreement between the results obtained by different authors. It has been fairly undisputed, however, that, in the presence of the coenzyme PLP and the substrate serine, the holoenzyme complex is tightly associated (Creighton & Yanofsky, 1966; Goldberg & Baldwin, 1967; Miles & Moriguchi, 1977), but the existence of a rather stable apoenzyme has been questioned recently by Miles & Moriguchi (1977), on the basis of gel filtration experiments.

Therefore as a prerequisite for any sound thermodynamic characterization of subunit interaction, the existence and the stoichiometry of the complex between α and β_2 subunits have to be established. The results obtained by thermometric titration and by calorimetric measurements of the saturation enthalpy suggest that, under the experimental conditions employed, only $\alpha_2\beta_2$ apoenzyme and α_2(β-PLP)$_2$ holoenzyme complexes are formed. The rather abrupt change from the practically linear increase of reaction enthalpy with increasing α-subunit concentration (Figure 1 and Tables I and II) at a molar ratio of $[\alpha]:[\beta_2] = 2:1$ excludes the occurrence of complexes other than $\alpha_2\beta_2$ composition. In addition, the lack of any appreciable curvature in the binding curve until about 80% saturation is indicative of tight binding. A more detailed binding study (Bartholmes & Teuscher, 1979) employing equilibrium dialysis, sedimentation velocity, and gel chromatography demonstrated the binding process to be consistent with either cooperativity between the two sites, if one assumes that prior to binding the two sites at the β_2 subunit are identical, or two intrinsically different binding sites for the α subunits. The values of the corresponding dissociation constants derived from fitting the binding parameters to an Adair type equation are at 25 °C $K_{D1} = 28 \pm 3$ μM and $K_{D2} = 0.8 \pm 0.1$ μM. Thus under the experimental conditions of the thermometric titration ($[\beta_2] = 8.37$ mg/mL = 188 μM sites before mixing 1:1 with the α subunit solution, 0.1 M pyrophosphate, pH 7.5), one has to expect practically stoichiometric binding, which renders the linear increase of ΔH with α

concentration understandable.

Saturation Enthalpies and Dependence on Temperature of Subunit Interaction. There are at least two important features demonstrated by the enthalpies, which appear to be general characteristics of reactions involving proteins. Inspection of the saturation enthalpies shows that at 25 °C the formation of the $\alpha_2\beta_2$ holoenzyme complex is favored by −22 kcal per mol of β_2 as against only −13 kcal per mol of β_2 pertaining to formation of the apoenzyme. Thus the presence of the coenzyme increases the enthalpic contribution to stability by almost a factor of two. This enhancement of the reaction enthalpy suffices to make it understandable that the dissociation constant of the holoenzyme at 25 °C is markedly smaller than that of the apoenzyme since practically the total difference in ΔH of −4.5 kcal/mol of β is reflected in the experimentally determined difference in Gibbs free energy of $-RT \ln 1000 = -4.1$ kcal/mol of β (Creighton & Yanofsky, 1966; Wilson & Crawford, 1965), 1000 being the approximate ratio of the holoenzyme to apoenzyme binding constant for association of α and β subunits.

The second feature of the subunit equilibrium of tryptophan synthase, which is worth stressing, is the remarkably large variation with temperature of the reaction enthalpies. Even if one refers the ΔC_p values not to the β_2 subunit but to the β protein, the 1150 cal K^{-1} (mol of β)$^{-1}$ for the holoenzyme and 1800 cal K^{-1} (mol of β)$^{-1}$ for the apoenzyme constitute ΔC_p changes, which are not found in equilibria between proteins and small ligands. Similar values occur only in interaction of proteins (Hearn et al., 1971; Bode et al., 1974; Hinz et al., 1979) and the magnitude of the ΔC_p changes is comparable but opposite in sign to the heat capacity changes associated with unfolding of proteins (Sturtevant, 1977). Potential sources of heat capacity changes concomitant with protein–protein and protein–ligand interaction, which of course are two names for the same process, have been considered by Sturtevant (1977) in his elegant analysis. A similar partition of the overall ΔC_p values into hydrophobic and vibrational contributions has not been attempted, however, since the number of systems for which data are available is not large enough to permit one to draw generalizations. Thus the phenomenon of interest worth keeping in mind is the pronounced dependence on temperature of the subunit interaction enthalpy, which this system has in common with other reactions involving protein–protein interactions.

A manifestation of the energetic linkage of equilibria occurring between a protein and various ligands is, in the case of tryptophan synthase, the influence of PLP on the temperature coefficients of the enthalpies. It is to the knowledge of the authors the first time that such a difference of the temperature coefficient of a reaction enthalpy originating from the presence of the coenzyme has been detected. This finding is in excellent agreement with the general qualitative interpretation of the negative ΔC_p values, as indicating a tightening of the structure due to reversal of hydrophobic hydration and a reduction in the number of easily excitable vibrational modes of the system. Reaction of the preformed $(\beta\text{-PLP})_2$ complex with the α subunits to yield the native $\alpha_2\beta_2$ structure apparently requires a smaller degree of conversion of soft internal modes to stiffer modes (Sturtevant, 1977) and of refolding of the protein structure into a more compact one than the analogous reaction between the apo-β_2 and the α subunits, since the β_2 subunit is already in a "tighter" configuration. That the "tighter" structure of the $(\beta\text{-PLP})_2$ complex leads also to higher stability has been demonstrated by scanning calorimetry studies (Hinz, 1979, unpublished results). Thus it appears to be justified to speak of a stabilization of the β_2 subunit induced by binding the coenzyme.

Association of Proton Absorption with Apo- and Holoenzyme Formation. Interactions between proteins are likely to change the energetic balance in the immediate surroundings of the contact areas but may also extend throughout the molecule, and therefore it is not surprising that the dissociation equilibria of ionizable groups are affected with a resulting proton release or absorption concomitant with the reaction. What is, however, remarkable is the experimental result of a difference in the number of protons absorbed at 35 °C, when the $\alpha_2\beta_2$ complex is formed, depending on the presence of the coenzyme PLP.

The change from two to one proton being absorbed during the association of the subunits at 35 °C, if the β_2 subunit has been incubated with PLP prior to the reaction, is a strong indication of an alteration of the internal equilibrium of the macromolecules in the holoenzyme compared with that in the apoenzyme. There is no a priori requirement that such an alteration is linked to a major conformational change. However, since this frequently appears to be the case, this matter is currently under investigation. Although it is unlikely that buffer effects play a role in shifting the H^+ equilibria, direct titrations will be performed at 25 and 35 °C to substantiate the change in the number of absorbed protons. An analogous question concerning the molecular implications responsible for the difference between the formation of the holoenzyme at 25 and 35 °C needs to be answered.

In summary, the calorimetric studies presented in this communication provide thermodynamic evidence for a pronounced linkage of coenzyme binding and subunit association. Both the association enthalpy of the α and β_2 subunits of tryptophan synthase and the temperature coefficient of the ΔH values are markedly affected by the presence of PLP. The results also demonstrate that, depending on the temperature, different numbers of protons are absorbed during complex formation, thus explicitly showing the coupling of the various equilibria.

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Subsite Interactions and Ribonuclease T_1 Catalysis: Kinetic Studies with ApGpC and ApGpU†

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ABSTRACT: The RNase T_1 catalyzed transesterification of ApGpC and ApGpU [to (ApG>p + C) and (ApG>p + U) products, respectively] was studied in steady-state kinetic experiments performed at 25 °C and 0.2 M ionic strength over the pH range 3–9. Values of k_{cat}/K_m and k_{cat} for these substrates were compared, as a function of pH, with those previously determined for GpC and GpU [Osterman, H. L., & Walz, F. G. (1978) *Biochemistry 17*, 4124]. The kinetic properties of ApGpN (N = C or U) substrates differed from those of GpN substrates in the following ways: (1) a dramatic attenuation of the pH dependence of k_{cat}/K_m; (2) no discrimination for C or U leaving nucleoside groups; and (3) an opposite pH dependence for k_{cat}. These results indicate that the adenosine moiety of the trimeric substrates binds with an enzyme subsite and that catalysis can proceed via three parallel reaction paths which are governed by apparent pK values of 5.2 and 7.7 in the enzyme–substrate complex. These paths are characterized by rate constants that are 130, 280, and 1670 s^{-1}, which predominate in the acidic, neutral, and basic pH ranges, respectively. Since catalysis of GpN substrates apparently utilizes a single reaction path [characterized by rate constants of 350 and 38 s^{-1} for GpC and GpU, respectively (Osterman & Walz, 1978)], it is concluded that the mechanism of catalysis for these trimeric and dimeric substrates is different. It is suggested that an active-site carboxylate group serves as a general base catalytic species in all cases except at high pH for ApGpN substrates, where an imidazole residue serves this function.

A complete understanding of substrate recognition and catalysis for depolymerizing enzymes requires studies of their behavior with multimeric substrates. This principle has been documented mainly in studies with proteases and carbohydrases (see Allen & Thoma (1976) for references), where the remote interaction of monomeric substrate units with enzyme subsites has been shown to influence the catalytic process. Similar studies with simple nucleases have been less systematic but have suggested the existence of electrostatic subsites for substrate phosphate groups in staphyllococcal nuclease (Cuatrecasas et al., 1968) and pancreatic RNase[1] A (Li & Walz, 1974; Mitsui et al., 1978). In addition, steady-state kinetic studies of RNase A with minimal RNA substrates (i.e., dinucleoside monophosphates CpN and UpN (N = A, C, G, U)) indicated a base group specific subsite interaction for adenine as the leaving (N) nucleoside group (Richards & Wyckoff, 1971). However, the existence of RNase A subsites for more complicated RNA substrates has not yet been investigated.

We have undertaken a systematic study of RNase T_1 with the final aim of understanding the detailed nature of its interaction with native RNA substrates and the relationship of these interactions to its catalytic action. RNase T_1 subsites were operationally identified in terms of possible enzyme binding with monomeric nucleoside and phosphate residues for the polymeric sequence: jN ... 2N-2p-1N-1p-G-p1-N1-

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[1] Abbreviations used: RNase, ribonuclease; Tris, 2-amino-2-hydroxy-1,3-propanediol; nucleotide designations follow the recommendations of the IUPAC–IUB commission as reported ((1970) *Biochemistry 9*, 4025).

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