Alberty, R. A. (1969) *J. Am. Chem. Soc. 91*, 3899–3903.
Balk, H. (1981) Thesis, Regensburg.
Balk, H., Merkl, I., & Bartholmes, P. (1981) *Biochemistry 20*, 6391–6395.
Bartholmes, P., Kirschner, K., & Gschwind, H.-P. (1976) *Biochemistry 15*, 4712–4717.
Bartholmes, P., Balk, H., & Kirschner, K. (1980) *Biochemistry 19*, 4527–4533.
Borri-Voltattorni, C., Orlacchio, A., Giartosio, A., Conti, F., & Turano, C. (1975) *Eur. J. Biochem. 53*, 151–160.
Crawford, I. P., Nichols, B. P., & Yanofsky, C. (1980) *J. Mol. Biol. 142*, 489–502.
Edsall, J. T., & Wyman, J. (1958) *Biophysical Chemistry*, p 453, Academic Press, New York.
Gaudin, C., Marty, B., Ragot, M., Sari, J. C., & Belaich, J. P. (1980) *Biochimie 62*, 741–746.
Giartosio, A., Salerno, C., Franchetta, F., & Turano, C. (1982) *J. Biol. Chem. 257*, 8163–8170.
Grenthe, J., Ots, H., & Ginstrup, O. (1970) *Acta Chem Scad. 24*, 1067–1080.
Gucker, F. T., Pickard, H. B., & Planck, R. W. (1939) *J. Am. Chem. Soc. 61*, 459–470.
Higgins, W., Miles, E. W., & Fairwell, T. (1980) *J. Biol. Chem. 255*, 512–517.
Hinz, H.-J. (1983) *Annu. Rev. Biophys. Bioeng. 12*, 285–317.
Hinz, H.-J., Shiao, D. D. F., & Sturtevant, J. M. (1971) *Biochemistry 10*, 1347–1352.
Hinz, H.-J., Gorbunoff, M. J., Price, B., & Timasheff, S. N. (1979) *Biochemistry 18*, 3084–3089.
Högberg-Raibaud, A., & Goldberg M. E. (1977a) *Proc. Natl. Acad. Sci. U.S. A. 74*, 442–446.
Högberg-Raibaud, A., & Goldberg, M. E. (1977b) *Biochemistry 16*, 4014–4020.
Johnson, R. J., & Metzler, D. E. (1970) *Methods Enzymol. 18A*, 433–471.
Kirschner, K., Wiscocil, R. L., Foehn, M., & Rezeau, L. (1975) *Eur. J. Biochem. 60*, 513–523.
Lane, A. (1983) *Eur. J. Biochem. 133*, 531–538.
Miles, E. W. (1979) *Adv. Enzymol. Relat. Areas Mol. Biol. 49*, 127–186.
Miles, E. W., & Moriguchi, M. (1977) *J. Biol. Chem. 252*, 6594–6599.
Niekamp, C. W., Hinz, H.-J., Jaenicke, R., Woenckhaus, C., & Jeck, R. (1980) *Biochemistry 19*, 3144–3152.
Orlacchio, A., Borri-Voltattorni, C., & Turano, C. (1980) *Biochem. J. 185*, 41–46.
Peterson, E. A., & Sober, H. A. (1954) *J. Am. Chem. Soc. 76*, 169–175.
Relimpio, A., Iriarte, A., Chlebowski, J. F., & Martinez-Carrion, M. (1981) *J. Biol. Chem. 256*, 4478–4488.
Ross, P. D., & Subramanian, S. (1981) *Biochemistry 20*, 3096–3102.
Schmid, F., Hinz, H.-J., & Jaenicke, R. (1976) *Biochemistry 15*, 3052–3059.
Schnackerz, K. D., & Bartholmes, P. (1983) *Biochem. Biophys. Res. Commun. 111*, 817–823.
Sturtevant, J. M. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2236–2240.
Tschopp, J., & Kirschner, K. (1980) *Biochemistry 19*, 4514–4521.
Weber, K., & Hinz, H.-J. (1976) *Rev. Sci. Instrum. 47*, 592–594.
Wiesinger, H., & Hinz, H.-J. (1980) *Therm. Anal.* [Proc. *Int. Conf.*], *6th*, 509–514.
Wiesinger, H., Bartholmes, P., & Hinz, H.-J. (1979) *Biochemistry 18*, 1979–1984.
Zetina, C. R., & Goldberg, M. E. (1980) *J. Biol. Chem. 255*, 4381–4385.
Zetina, C. R., & Goldberg, M. E. (1982) *J. Mol. Biol. 157*, 133–148.

Energetic Adaptation of Ligand Binding to Subunit Structure of Tryptophan Synthase from *Escherichia coli*†

Heinrich Wiesinger and Hans-Jürgen Hinz*

ABSTRACT: The binding of indole and L-serine to the isolated α and β_2 subunits and the native $\alpha_2\beta_2$ complex of tryptophan synthase from *Escherichia coli* was investigated by direct microcalorimetry to reveal the energetic adaptation of ligand binding to the subunit structure of a multienzyme complex. In contrast to the general finding that negative heat capacity changes are associated with ligand binding to proteins, complex formation of indole and the α subunit involves a small positive change in heat capacity. This unusual result was considered as being indicative of a loosening of the protein structure. Such an interpretation is in good agreement with results of chemical accessibility studies (Freedberg & Hardman, 1971). Whereas the thermodynamic parameters of indole binding are not influenced by the subunit interaction, the large negative change in heat capacity of −6.5 kJ/(K·mol of β_2) measured for the binding of L-serine to the isolated β_2 subunit disappears completely when serine interacts with the tetrameric complex. These data demonstrate that the energy transduction pattern and therefore the functional roles of the substrates indole and L-serine vary strongly with the subunit structure of tryptophan synthase.

Specificity of coenzyme and substrate binding and catalytic efficiency of enzymes are highly correlated properties. Energy transduction upon complex formation with the specific ligands induces the energetic and structural changes necessary for the enzyme to reach the activated complex at physiological temperatures. Due to the linkage of all binding equilibria interactions between subunits of multimeric enzymes can be expected to influence the energetic pattern of substrate binding considerably (Pettigrew et al., 1982; Wiesinger et al., 1979).

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The interdependence between ligand binding, protein–protein interaction, and catalytic activity can be conveniently studied on multifunctional enzymes, particularly if the various catalytic properties are associated with different subunits. An intriguing system of this type is the bienzyme complex tryptophan synthase from *Escherichia coli* (Miles, 1979). The enzyme catalyzes the last step in the biosynthesis of L-tryptophan.

indoleglycerol phosphate + L-serine =
L-tryptophan + indole + H_2O (1)

Tryptophan synthase is an $\alpha_2\beta_2$ tetramer which can be dissociated into two α and the β_2 subunits. An active $\alpha_2\beta_2$ complex can also be reconstituted from the α and β_2 subunits. Each subunit catalyzes part of the overall reaction 1. The cleavage of indoleglycerol phosphate is catalyzed by the α subunit (eq 2), and the condensation of indole and L-serine

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

indole + L-serine = L-tryptophan + H_2O (3)

is catalyzed by the β_2 subunit with the participation of the coenzyme pyridoxal 5′-phosphate (PLP) (eq 3). It is a characteristic feature of the bifunctional $\alpha_2\beta_2$ complex that both partial reactions 2 and 3 are up to 100-fold more efficiently catalyzed by the tetrameric species than by the α and β_2 subunits alone (Yanofsky & Crawford, 1972). Therefore, it must be assumed that the energetic and structural changes concomitant with subunit association are responsible for the higher catalytic activity of the $\alpha_2\beta_2$ complex as compared with that of the separated α and β_2 subunits.

The energetic and indirectly also the structural changes can be uniquely investigated by direct microcalorimetric measurements of the energies associated with $\alpha_2\beta_2$ complex formation from the α and β_2 subunits. Linkage of subunit association with ligand binding will be reflected in the variation of energy parameters in the presence of various ligands. The large influence of the coenzyme PLP on the thermodynamic parameters of subunit association has been demonstrated in a previous study (Wiesinger et al., 1979). It has been found that proton flux, reaction enthalpies, and heat capacity changes, which are indicative of structural changes of the macromolecule (Sturtevant, 1977; Hinz et al., 1981; Hinz, 1983), are very different for apoenzyme and holoenzyme formation from the isolated α and β_2 and α and $(\beta$–PLP$)_2$ subunits, respectively.

Since interaction with the coenzyme is only one step in the series of structural and energetic transformations which the enzyme undergoes on its way to catalytic activity, studies were undertaken to characterize the energetic changes involved in complex formation between the enzyme and two other ligands, L-serine and indole. The binding characteristics of these two substrates can be assumed to be quite different. Indole is formed on the α subunit, transported to the β subunit by a yet unknown mechanism, and there condensed with L-serine to form L-tryptophan. The fact that indole is product and substrate, respectively, of both partial reactions catalyzed by the individual subunits shows that this substrate must be able to interact with both α and β_2 subunits. In contrast, L-serine binds only to the β_2 subunit. It interacts noncovalently with the β_2 subunit and in addition replaces the covalent Schiff base between PLP and the enzyme.

The present investigations are intended to quantify the various interactions of these two ligands and to reveal the energetic and structural differences between the isolated α and β_2 subunits and the same subunits in the native $\alpha_2\beta_2$ complex. These differences reflect the energetic adaptation of ligand binding to the bifunctionality of the $\alpha_2\beta_2$ tetramer of tryptophan synthase.

Materials and Methods

The α and β_2 subunits of tryptophan synthase were purified and stored as described previously (Kirschner et al., 1975b; Bartholmes et al., 1976). The concentration of α protein was determined at 278 nm by using an extinction coefficient of $E^{0.1\%} = 0.58$ cm^2 mg^{-1}. The concentration of holo β_2 protein was determined at 290 nm after diluting the protein 1:20 in 0.1 M NaOH by using an extinction coefficient $E^{0.1\%} = 0.75$ cm^2 mg^{-1}. As at pH 13 the coenzyme PLP shows absorption bands at 388 and 290 nm, the contribution of this latter extinction has to be corrected for according to the equation

$$[\text{holo } \beta_2] = \frac{(E_{290} - 0.091E_{388}) \times 20}{0.75} \quad \text{(mg/mL)}$$

The tetrameric complex was reconstituted by adding an excess of α subunits to a solution of the β_2 dimer (Wiesinger et al., 1979). The concentration of indole was determined at 269 nm by using an extinction coefficient of 5.75×10^3 M^{-1} cm^{-1}.

All experiments were performed in 0.1 M sodium pyrophosphate buffer adjusted with HCl to pH 7.5. For measurements in the presence of coenzyme the buffer contained 0.02 mM PLP. PLP was purchased from Serva, Heidelberg; all other chemicals were obtained from Merck, Darmstadt, and were of reagent-grade quality.

Absorption measurements were performed in a Varian Cary 118 spectrophotometer, and pH values were adjusted at the temperature of the experiment by using a digital pH meter (WTW, Weilheim). The microcalorimeter (Weber & Hinz, 1976) was operated in the pulsed flow mode with pulse duration between 20 and 50 s, corresponding to 0.15 and 0.37 mL of solution, respectively. All molar enthalpies have been referred to a molecular weight of 29 000 and 89 000 for the α and β_2 subunit, respectively, and have been corrected for dilution effects of the components. The calorimeter was calibrated by using the well-known heats of neutralization of water and dilution of sucrose (Grenthe et al., 1970; Gucker et al., 1939). The error within a single determination of a ΔH value consisting of three to seven measurements is about 10% and is not listed in the tables. The binding curves in Figure 1 were calculated according to an Adair type model by using eq 4 and 5. $K_{D,1}$ and $K_{D,2}$ are the dissociation constants of

$$\Delta H = \frac{2\Delta H_{A,1}L/K_{D,1} + (\Delta H_{A,1} + \Delta H_{A,2})L^2/(K_{D,1}K_{D,2})}{1 + 2L/K_{D,1} + L^2/(K_{D,1}K_{D,2})} \quad (4)$$

$$[(L + 2K_{D,2} + 2E_0 - L_0)L + K_{D,1}K_{D,2} + 2E_0K_{D,2} - 2L_0K_{D,2}]L - L_0K_{D,1}K_{D,2} = 0 \quad (5)$$

binding sites 1 and 2, and $\Delta H_{A,1}$ and $\Delta H_{A,2}$ are the corresponding assocation enthalpies for saturing indole concentrations. L refers to the free indole concentration, and L_0 and P_0 are the analytical indole and α subunit concentrations. ΔH is the observed association enthalpy. Iterative least-squares procedures were used to obtain the free ligand concentration L from eq 5. That value was then employed in eq 4 to approximate the experimental ΔH by treating $\Delta H_{A,1}$ and $\Delta H_{A,2}$ as adjustable parameters.

Results

(*A*) *Binding of Indole to the Isolated α and Holo β_2 Subunits.* Due to the low binding constants of indole to the α and

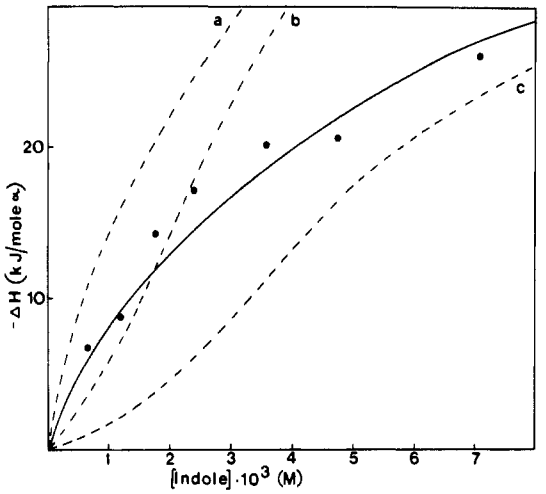

FIGURE 1: Enthalpimetric titration of the α subunit of tryptophan synthase with indole at 25 °C, pH 7.5; $[\alpha] = 2 \times 10^{-4}$ M after mixing. Curve calculated with $K_D = 5$ mM, $\Delta H_{A,max} = -46$ kJ/mol of α, and $n = 1$. Dotted curves calculated with the following (see Materials and Methods): (a) $K_D = 5$ mM, $\Delta H_{A,max} = -46$ kJ/mol of α and $n = 2$; (b) $K_{D,1} = 18$ mM and $\Delta H_{A,1} = -41.8$ kJ/mol; $K_{D,2} = 1.5$ mM and $\Delta H_{A,2} = -4.2$ kJ/mol; (c) $K_{D,1} = 18$ mM and $\Delta H_{A,1} = -4.2$ kJ/mol; $K_{D,2} = 1.5$ mM and $\Delta H_{A,2} = -41.8$ kJ/mol.

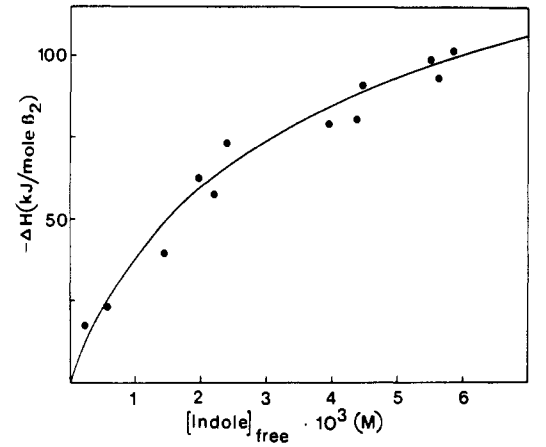

FIGURE 2: Enthalpimetric titration of the holo β_2 subunit of tryptophan synthase with indole at 25 °C, pH 7.5; the data shown are from several experiments with different enzyme concentrations; therefore, they were drawn as a function of the concentration of free indole. Curve calculated with $K_D = 2.4$ mM, $\Delta H_{D,max} = 136$ kJ/mol of β_2, and $n = 20/\beta_2$.

β_2 subunits of tryptophan synthase, it was possible to perform calorimetric titrations at 10, 25, and 35 °C. The solubility of indole in the aqueous buffer solutions used is approximately 16 mM; since ligand and enzyme solutions are mixed 1:1 in the calorimeter, the highest indole concentrations reached in the experiments were around 7–8 mM; no saturation enthalpies could be measured under these conditions. The curves shown in Figures 1 and 2 were calculated by using the K_D values and the number n of binding sites obtained from least-squares fits of the linear function $K_D/(1 - \alpha) = L_0/(\alpha - nP_0)$, which can be derived from the mass law expression under the assumption of equivalent and independent binding sites. K_D is the dissociation constant; L_0 is the initial ligand concentration; P_0 is the initial enzyme concentration; n is the number of binding sites per enzyme; α is defined by the ratio $\Delta H/\Delta H_{max}$. A good first approximate value of ΔH_{max} can be estimated from the titration experiments; in an iteration procedure the parameters K_D, n, and ΔH_{max} are varied until the calculated curve fits the experimental data. Although it formally is a three-parameter fit, there is not much freedom in varying ΔH_{max}, which is rather accurately obtained from extrapolation of the experimental binding curves.

Table I: Enthalpy of Binding of Indole to the α Subunit of Tryptophan Synthase at 25 °C, pH 7.5, as a Function of Indole Concentration[a]

[indole] (mM)	$-\Delta H$ (kJ/mol of α)	[indole] (mM)	$-\Delta H$ (kJ/mol of α)
7.1	25.9	1.8	14.2
4.7	20.5	1.2	8.8
3.6	20.1	0.7	6.7
2.4	17.1		

[a] $[\alpha] = 2 \times 10^{-4}$ M; values refer to concentrations after mixing.

Table II: Enthalpy of Binding of Indole to the α Subunit of Tryptophan Synthase at 35 °C, pH 7.5, as a Function of Indole Concentration[a]

[indole] (mM)	$-\Delta H$ (kJ/mol of α)	[indole] (mM)	$-\Delta H$ (kJ/mol of α)
6.0	18.0	1.7	10.0
4.0	17.1	1.2	5.9
3.0	11.7		

[a] $[\alpha] = 1.5 \times 10^{-4}$ M; values refer to concentrations after mixing.

Table III: Enthalpy of Binding of Indole to the Holo β_2 Subunit of Tryptophan Synthase at 10 °C, pH 7.5, as a Function of Indole Concentration[a]

[indole] (mM)	$-\Delta H$ (kJ/mol of β_2)	[indole] (mM)	$-\Delta H$ (kJ/mol of β_2)
6.0	92.5	1.5	46.9
3.5	72.0	0.6	25.1
3.1	66.5	0.4	32.6
1.95	61.1	0.3	25.1
1.9	51.5		

[a] $[\beta_2] = 4.1 \times 10^{-5}$ M; values refer to concentrations after mixing.

Table IV: Enthalpy of Binding of Indole to the Holo β_2 Subunit of Tryptophan Synthase at 35 °C, pH 7.5, as a Function of Indole Concentration[a]

[indole] (mM)	$-\Delta H$ (kJ/mol of β_2)	[indole] (mM)	$-\Delta H$ (kJ/mol of β_2)
5.9	77.4	1.5	31.0
4.7	76.6	1.2	25.9
3.0	58.6	0.6	28.5
2.0	40.2	0.3	15.1

[a] $[\beta_2] = 5.0 \times 10^{-5}$ M; values refer to concentrations after mixing.

The variation with concentration of the binding enthalpy of indole to the α subunit at 25 and 35 °C is given in Tables I and II. Each value for ΔH is the mean of three or four measurements. Each titration experiment is best represented by a curve calculated on the assumption of a single binding site and the thermodynamic parameters given in Table V. As can be seen from Figure 1 the enthalpimetric data can be represented neither by two identical binding sites nor by two different binding sites as reported in the literature (Weischet & Kirschner, 1975). The dissociation constant of 5 mM at 25 °C resulting from our measurements is approximately the geometrical mean of the values reported by Weischet and Kirschner. The binding of indole is slightly stronger at the higher temperature ($K_D = 3.5$ mM), and the decrease in binding enthalpy from −46.0 to −29.3 kJ/mol with increasing temperature corresponds to a positive change in heat capacity of 1.7 kJ/(K·mol).

Similar titration experiments were performed at 10, 25, and 35 °C employing indole and the β_2–PLP_2 complex, i.e., the

Table V: Thermodynamic Parameters of Binding of Indole to Subunits of Tryptophan Synthase at pH 7.5

enzyme	T (°C)	$-\Delta H$ (kJ/mol of bs)[a]	$-\Delta G$ (kJ/mol of bs)[a]	ΔS [J/(K·mol of bs)][a]	n[c]
β_2	10	6.0 ± 0.4[b]	15.9 ± 0.4[b]	34.7	10 ± 1.5
β_2	25	6.8	15.1	27.2	10 ± 1.5
β_2	35	7.3	14.2	22.2	10
α	25	46.0	13.0	−109.2	1
α	35	29.3	14.6	−48.1	1

[a] bs = binding site. [b] These variations are possible without significant changes in the fitted curves. [c] n = number of binding sites per β and α chain, respectively.

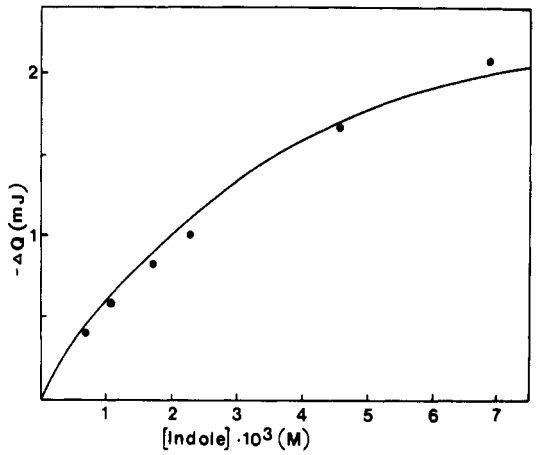

FIGURE 3: Enthalpimetric titration of the holo $\alpha_2\beta_2$ complex of tryptophan synthase with indole at 25 °C, pH 7.5; calculation of the curve is described in the text; $[\alpha] = 7.1 \times 10^{-5}$ M; $[\beta_2] = 3.4 \times 10^{-5}$ M in 0.185 mL after mixing.

β_2 subunit presaturated with the coenzyme. A typical experiment is shown in Figure 2 for 25 °C, and the ΔH values determined at 10 and 35 °C are listed in Tables III and IV. The titration curves can only be fitted when a large number n of unspecific binding sites (n = 10 per protein chain) at all temperatures and a small enthalpy per binding site are employed. The resulting thermodynamic parameters are summarized in Table V.

(B) Binding of Indole to the $\alpha_2\beta_2$–PLP$_2$ Complex. The enthalpy of binding of indole to the holo $\alpha_2\beta_2$ complex of tryptophan synthase was measured in the calorimeter as a function of the ligand concentration. An excess of α protein was used in all experiments to achieve complex formation. Therefore, an enthalpy value per binding site cannot be calculated. However, the following procedure was adopted to compare the energetics of binding of indole to the subunits and to the native complex: it was assumed that indole binding is independent of complex formation and that the protein–protein interaction does not influence the binding parameters, which also implies that the enthalpy of interaction of α + β_2(PLP)$_2$ is the same as that for the subunits presaturated with indole. On the basis of the molar enthalpies of binding of indole to the individual subunits (see section A), the dissociation constants, and the known molar quantities of subunits in the mixing cell of the calorimeter, one can calculate a value ΔQ for the expected heat effect and compare it with the heat actually exchanged in the calorimetric cell. As can be seen in Figures 3 and 4 the experimentally measured ΔQ values are well represented by the calculated curves within experimental error. This result verifies the original assumption that the binding enthalpies of indole are not influenced by the protein–protein interaction between α and β subunits of tryptophan synthase.

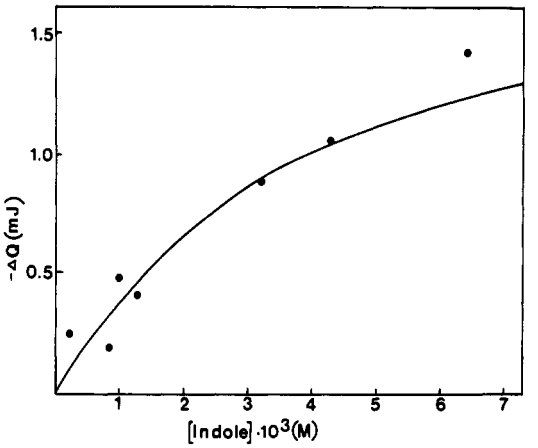

FIGURE 4: Enthalpimetric titration of the holo $\alpha_2\beta_2$ complex of tryptophan synthase with indole at 35 °C, pH 7.5; calculation of the curve is described in the text; $[\alpha] = 9 \times 10^{-5}$ M; $[\beta_2] = 2.25 \times 10^{-5}$ M in 0.148 mL after mixing.

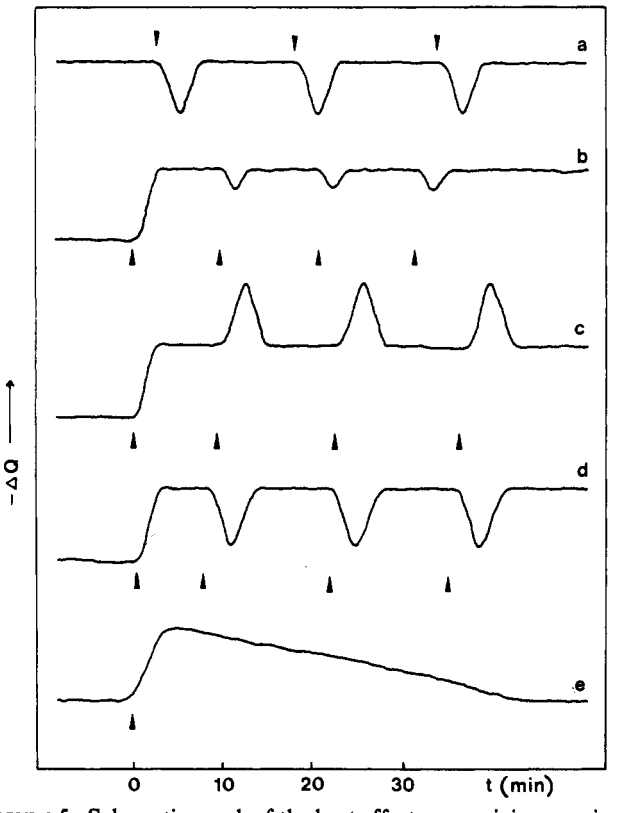

FIGURE 5: Schematic graph of the heat effect upon mixing L-serine with (a) holo β_2 at 15 °C, (b) holo β_2 at 25 °C, (c) holo β_2 at 35 °C, (d) holo $\alpha_2\beta_2$ at 25 or 35 °C, and (e) holo β_2 at 35 °C (low concentration of L-serine). (▲) Successive mixing in the calorimeter cell; pulse duration 50 s; for explanation of the steady-state exothermic signal in (a–d), see footnote 2.

(C) Binding of L-Serine to the β_2–PLP$_2$ and the $\alpha_2\beta_2$–PLP$_2$ Complexes. The calorimetric curves of binding L-serine to the holo β_2 subunit are very complex because the protein is enzymatically active in the presence of this ligand. Thus, binding occurs concomitant with deamination of L-serine which yields pyruvate and ammonia (Crawford & Ito, 1964). The heat exchange pattern is illustrated in Figure 5 as a function of time. A qualitative description of the various experiments is the following:[1] when β_2–PLP$_2$ is mixed with L-serine in the cal-

[1] In all experiments a possible heat effect stemming from the interaction of L-serine with PLP in the buffer was compensated for by mixing identical solutions without enzyme in a reference cell.

Table VI: Enthalpy of Binding of L-Serine to Holo-Tryptophan Synthase at pH 7.5

enzyme	T (°C)	ΔH (kJ/mol of β_2)
β_2	15	105.4 ± 6.7[a]
β_2	25	33.5 ± 5.0
β_2	35	−24.7 ± 1.7
$\alpha_2\beta_2$	25	105.0 ± 7.1
$\alpha_2\beta_2$	35	105.0 ± 7.5

[a] Standard deviation of at least three measurements.

orimeter at 15 °C, only a fast endothermic reaction can be detected (curve a). Performing the same experiment at 25 and 35 °C results in a very different heat exchange pattern. At 25 °C a slow exothermic reaction reaches a steady-state plateau after the first mixing. Every additional mixing (curve b) is followed by a fast endothermic reaction.[2] At 35 °C the slow exothermic reaction is still manifested in the plateau (curve c); however, additional mixings of the β_2–PLP_2 complex with L-serine produce now fast exothermic reactions. When instead of β_2–PLP_2 the native $\alpha_2\beta_2$–PLP_2 tetramer is used, heat exchange pattern of the type d were detected, which did not change with temperature. At 25 and 35 °C mixing of L-serine with $\alpha_2\beta_2$–PLP_2 results in the identical fast endothermic reaction on top of the exothermic plateau. Finally, to characterize the slow reaction, β_2–PLP_2 was reacted at 35 °C with a solution having only low serine concentration. The heat flux as a function of time is shown in curve e of Figure 5. The reaction lasts approximately 40 min, and the heat change involved is negative.

It has been shown by an independent set of experiments in a batch calorimeter that the slow deamination of L-serine catalyzed by the holo β_2 subunit of tryptophan synthase results in an enthalpy of −7.3 kJ/mol of serine (H. Wiesinger and H.-J. Hinz, unpublished results). The solutions after the experiments in the flow calorimeter were analyzed by enzymatic methods, and the detection of pyruvate indicated that indeed the substrate L-serine was converted into its deamination products. On the basis of these results we interpret the calorimetric experiments in the following manner:

At 15 °C the deamination in the presence of β_2–PLP_2 is too slow and cannot be detected with a significant heat effect. The enthalpies of binding can be calculated from the peak area without interference by a steady-state heat effect. At 25 and at 35 °C the binding of serine is manifested by the endothermic and exothermic peaks on top of the exothermic plateau of the slow deamination reaction, and the deamination is not completely inhibited in the holo $\alpha_2\beta_2$ complex as reported by Lane & Kirschner (1983b). By use of equilibrium constants from the literature (Faeder & Hammes, 1970, 1971; Lane & Kirschner, 1983a), the extent of binding could be calculated. By application of the van't Hoff equation, we obtained the equilibrium constants for the other temperatures.

All thermodynamic parameters for serine binding are summarized in Table VI. It is worth noting that interaction of β_2–PLP_2 with serine is characterized by a negative heat capacity change of −6.5 kJ/(K·mol of β_2), while binding to the $\alpha_2\beta_2$ complex is not associated with a ΔC_p.

The consistency of our interpretation is supported by the following consideration: Curve e of Figure 5 shows an experiment where a solution of large enzyme concentration is mixed with a serine solution of low concentration. Therefore, the enthalpy calculated from the area under the curve comprises the enthalpy of binding of serine in addition to the enthalpy of the catalytic deamination reaction. After the enthalpy of binding as determined from curve c of Figure 5 is subtracted, the resulting enthalpy value should be identical with the ΔH of the enzymatic reaction as obtained in the independent batch experiments in the presence of catalytic amounts of the enzyme, where enthalpies of binding do not interfere. The ΔH value obtained by the procedure is −7.1 kJ/mol of serine which is identical within experimental error with the −7.3 kJ/mol of serine determined for the catalytic reaction in the batch calorimeter.

Discussion

Free Energies and Enthalpies of Indole Binding to the Subunits and the Tetrameric Complex. Although the bienzyme complex tryptophan synthase from *E. coli* has been well characterized with respect to the kinetics and the mechanism of ligand binding (Miles, 1979), the thermodynamic description of the enzyme has been incomplete since only equilibrium constants, i.e., the Gibbs energies, have been known so far. This lack of energetic data and the fact that protein–protein interactions between the α and the β subunits have been demonstrated to have a pronounced effect on the thermodynamics of coenzyme binding (Wiesinger & Hinz, 1984) were the rationale for investigating the binding of indole and L-serine to the subunits and the native complex by microcalorimetric methods. Changes in energy and particularly changes in heat capacity reflect more readily changes in the reaction mechanism than the Gibbs energies. $\Delta G°$ values are always composite quantities of frequently compensating energy and entropy changes and therefore not suitable for molecular interpretation. It is one of the pertinent results of this study to have demonstrated that the energy transduction on complex formation and the resulting structural adjustments of the α and β subunits of tryptophan synthase depend critically on the state of association of the subunits.

Differences in the energetic parameters of indole binding to the isolated subunits and to the native tetrameric complex were determined as a function of temperature (Table V). Obviously indole can interact with a high number of identical binding sites on the β_2 subunit (10 per polypeptide chain), each of which at 25 °C contributes the same small amount of −6.7 kJ/mol to the overall enthalpy change of −136 kJ/mol of β_2. The same number of binding sites with a dissociation constant of 11.6 mM was determined by Weischet & Kirschner (1976). Analysis of the thermometric titrations performed in this study yielded a somewhat smaller dissociation constant (2.4 mM); however, our experiments as well as the results of the equilibrium dialysis studies did not find evidence for a single binding site with high affinity for indole, as was suggested by a K_m value of 4 μM by Heilmann (1978).

No unequivocal description of the stoichiometry of indole binding to the α subunit has been reported in the literature. Freedberg & Hardman (1971) analyze their binding data with a single binding site and a dissociation constant of 3.4 mM. On the other hand, from equilibrium dialysis experiments with radioactive indole, it was concluded that indole binds weakly in the active center of the α subunit (K_D = 18 mM) but that a further binding site with a K_D of 1.8 mM exists (Weischet & Kirschner, 1976). It is not clear, however, what the role of this extra binding site with higher affinity should be if one considers the fact that during the physiological reaction indole has to leave the α subunit and has to interact with the β_2 protein. Our calorimetric titrations can only be evaluated with

[2] Due to the length of the tubing behind the mixing cell, the solution of the still reacting components is not pushed out of the thermopile upon several successive mixings (Weber & Hinz, 1976). Therefore, an exothermic steady-state signal is observed.

a single binding site, the dissociation constant of which at 25 °C (5 mM) is the geometrical mean of the values cited. The result therefore supports the data reported by Freedberg & Hardman (1971).

The small temperature dependence of the reaction enthalpies allows partitioning of the free energies into enthalpic and entropic contributions. The reaction of indole with β_2–PLP_2 is driven by the change in entropy, whereas in the case of the α subunit enthalpic contributions dominate (Table V). Therefore, the idea of hydrophobic interaction holds for the indole–β_2 reaction, although the stability of the complex decreases slightly with temperature.

Changes in Heat Capacity during Indole Binding to the α Subunit. The change in heat capacity during a reaction involving biological macromolecules, which is experimentally determined as the temperature dependence of the reaction enthalpy, can be interpreted in molecular terms as indicating structural events in the macromolecule (Sturtevant, 1977; Hinz, 1983). Large positive changes in heat capacity were generally found for the unfolding of small globular proteins (Privalov, 1979) and rationalized by the higher number of easily excitable degrees of freedom and an increase in the hydrophobic hydration in the unfolded protein. Thus, negative changes in heat capacity resulting from the interaction of a protein with a ligand imply a tighter structure of the protein after the reaction.

A change in heat capacity of 1.7 kJ/(K·mol of α) was determined in our calorimetric experiments for the interaction of indole with the α subunit. It is the first time that a positive sign of this parameter is reported for ligand binding to a protein. Since recent X-ray studies have confirmed the interpretation of ΔC_p that negative heat capacity changes can be rationalized as resulting from structural changes of the macromolecule which lead to a tighter conformation (Janin & Wodak, 1983), we infer that the positive heat capacity change found for binding of indole to the α subunit indicates local loosening or partial unfolding of the protein upon complex formation. Independent experiments reported in the literature support this interpretation. Freedberg & Hardman (1971) demonstrated that in the absence of indole only cysteines-80 and -112 are accessible for the sulfhydryl reagent *N*-ethylmaleimide whereas in solutions of high indole molarity cysteine-153 can also be modified. Reactivity of cysteine-153 induced by indole is indicative of a conformational change in the α subunit which results also in decreased stability as shown by the higher sensitivity against thermal inactivation (Kirschner & Wiscocil, 1972). Interpretation of the calorimetrically obtained ΔC_p values therefore is in good agreement with evidence obtained by independent physical–chemical methods.

During the physiological reaction, indole, being the product of the fist half-reaction, has to dissociate from its binding site on the α subunit, which is not juxtaposed to the active center on the β_2 subunit, as was demonstrated recently (Lane, 1983). Thus, tightening and loosening of the structure of the α subunit are a cyclic process in the course of the catalytic overall reaction of tryptophan synthase. In view of the general finding that changes in heat capacity are associated with coenzyme and substrate binding, reversible structural changes of an enzyme must be postulated to be a characteristic feature of the catalytic mechanism. The thermodynamic evidence gains strong support from recent high-resolution X-ray analysis of various enzyme systems (Janin & Wodak, 1983).

Characteristics of the Energy Parameters for Serine Binding to the Isolated β_2–PLP_2 Subunit and the Native $\alpha_2\beta_2$–PLP_2 Tetramer. The thermodynamic data and particularly the interpretation of the characteristic heat capacity changes associated with binding of L-serine to tryptophan synthase are fully consistent with results obtained by Faeder & Hammes (1970, 1971) from kinetic studies. The occurrence of a negative change in heat capacity of −6.5 kJ/(K·mol of β_2) upon serine binding to β_2–PLP_2 and the absence of a ΔC_p for complex formation of serine with $\alpha_2\beta_2$–PLP_2 imply that only binding of serine to the isolated holo β_2 subunit involves structural rearrangements which can be described as tightening.

Applying methods of fast kinetics, Faeder & Hammes (1970) had shown that the β_2–PLP_2 as well as the β_2–PLP_2–Ser_2 complex can exist in two different conformational states. Therefore, it is necessary that a structural isomerization of the protein occurs as a result of serine binding. In analogous experiments with the $\alpha_2\beta_2$–PLP_2 complex no isomerization processes were detected. It can therefore be concluded that the native $\alpha_2\beta_2$–PLP_2 complex is already "frozen" in a conformation which is adapted to the interaction with the substrate serine (Faeder & Hammes, 1971).

The binding of L-serine to the β protein involves noncovalent interactions and covalent schiff base formation. The bond between the formyl group of the coenzyme pyridoxal 5′-phosphate and lysine-86 is broken and replaced by a similar Schiff base linkage between PLP and the amino acid L-serine. The net effect on the overall enthalpy of this process should be small, especially since the heat effects of Schiff base formation alone are vanishing at 25 °C, pH 7.5, as was shown by studies on model compounds (Wiesinger & Hinz, 1980). The coenzyme PLP remains attached to the protein by noncovalent forces after the imine linkage to lysine-86 is broken. Therefore, there is no contribution to the overall enthalpy from a dissociation of the coenzyme. Another interaction is, however, reflected in the calorimetrically observed ΔH. Tanizawa & Miles (1983) showed that the carboxylic group of L-serine binds to arginine-148 of the β protein, where a cluster of basic residues facilitates the anion binding. This process should result in a considerable positive contribution to the measured reaction enthalpy.

Conclusion

Enthalpimetric titrations of indole to the holo $\alpha_2\beta_2$ complex can be described by the assumption that protein–protein interaction between the subunits does not influence the binding process. The same thermodynamic parameters can be used for calculating the binding curves of the individual subunits and the tetrameric complex. The finding of an increased number of binding sites with low affinity in the complex (Weischet & Kirschner, 1976) was not verified. Therefore, indole seems to be the only ligand that binds to the subunits and the complex in the same manner. Differences in stability were found for all other ligands, for L-serine (Faeder & Hammes, 1970, 1971), for tryptophan (Faeder & Hammes, 1970), and for indoleglycerol phosphate (Kirschner et al., 1975a). Differences in the binding enthalpies and their temperature dependence were found in calorimetric experiments for pyridoxal 5′-phosphate (Wiesinger & Hinz, 1984) and for L-serine, as reported in this study. It can therefore be concluded that the unique role of indole as substrate for both subunits and as an intermediate compound in the reaction sequence of tryptophan synthase is clearly reflected in its energetic behavior.

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Registry No. Indole, 120-72-9; L-serine, 56-45-1; tryptophan synthase, 9014-52-2.

References

Bartholmes, P., Kirschner, K., & Gschwind, H.-P. (1976) *Biochemistry 15*, 4712–4717.
Crawford, I. P., & Ito, J. (1964) *Proc. Natl. Acad. Sci. U.S.A. 51*, 390–397.
Faeder, E. J., & Hammes, G. G. (1970) *Biochemistry 9*, 4043–4049.
Faeder, E. J., & Hammes, G. G. (1971) *Biochemistry 10*, 1041–1045.
Freedberg, W. P., & Hardman, J. K. (1971) *J. Biol. Chem. 246*, 1449–1456.
Grenthe, F., Ots, H., & Ginstrup, O. (1970) *Acta Chem. Scand. 24*, 1067–1080.
Gucker, F. T., Pickard, H. B., & Planck, R. W. (1939) *J. Am. Chem. Soc. 61*, 459–470.
Heilmann, H. D. (1978) *Biochim. Biophys. Acta 522*, 614–624.
Hinz, H.-J. (1983) *Annu. Rev. Biophys. Bioeng. 12*, 285–317.
Hinz, H.-J., Cossmann, M., & Beyreuther, K. (1981) *FEBS Lett. 129*, 246–248.
Janin, J., & Wodak, S. J. (1983) *Prog. Biophys. Mol. Biol. 42*, 21–78.
Kirschner, K., & Wiscocil, R. L. (1972) in "*Protein–Protein Interactions*" (Jaenicke, R., & Helmreich, E., Eds.) pp 245–268, Springer, Heidelberg.
Kirschner, K., Weischet, W., & Wiscocil, R. L. (1975a) in *Protein–Ligand Interactions* (Sund, H., & Blauer, D., Eds.) pp 27–44, de Gruyter, Berlin.
Kirschner, K., Wiscocil, R. L., Foehn, M., & Rezeau, L. (1975b) *Eur. J. Biochem. 60*, 513–523.
Lane, A. (1983) *Eur. J. Biochem. 133*, 531–538.
Lane, A., & Kirschner, K. (1983a) *Eur. J. Biochem. 129*, 561–570.
Lane, A., & Kirschner, K. (1983b) *Eur. J. Biochem. 129*, 571–582.
Miles, E. W. (1979) *Adv. Enzymol. Relat. Areas Mol. Biol. 49*, 127–186.
Pettigrew, D. W., Romeo, P. H., Tsapis, A., Thillet, J., Smith, M. L., Turner, B. W., & Ackers, G. K. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 1849–1853.
Privalov, P. L. (1979) *Adv. Protein Chem. 33*, 167–241.
Sturtevant, J. M. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2236–2240.
Tanizawa, K., & Miles, E. W. (1983) *Biochemistry 22*, 3594–3603.
Weber, K., & Hinz, H.-J. (1976) *Rev. Sci. Instrum. 47*, 592–594.
Weischet, W. O., & Kirschner, K. (1976) *Eur. J. Biochem. 64*, 313–320.
Wiesinger, H., & Hinz, H.-J. (1980) *Therm. Anal.* [*Proc. Int. Conf.*], *6th*, 509–514.
Wiesinger, H., & Hinz, H.-J. (1984) *Biochemistry* (preceding paper in this issue).
Wiesinger, H., Bartholmes, P., & Hinz, H.-J. (1979) *Biochemistry 18*, 1979–1984.
Yanofsky, C., & Crawford, J. P. (1972) *Enzymes*, *3rd Ed. 7*, 1–31.

Kinetics and Subunit Interaction of the Mannitol-Specific Enzyme II of the *Escherichia coli* Phosphoenolpyruvate-Dependent Phosphotransferase System†

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ABSTRACT: Purified mannitol-specific enzyme II (EII^{mtl}), in the presence of the detergent Lubrol, catalyzes the phosphorylation of mannitol from P–HPr via a classical ping-pong mechanism involving the participation of a phosphorylated EII^{mtl} intermediate. This intermediate has been demonstrated by using radioactive phosphoenolpyruvate. Upon addition of mannitol, at least 80% of the enzyme-bound phosphoryl groups can be converted to mannitol 1-phosphate. The EII^{mtl} concentration dependence of the exchange reaction indicates that self-association is a prerequisite for catalytic activity. The self-association can be achieved by increasing the EII^{mtl} concentration or at low concentrations of EII^{mtl} by adding HPr or bovine serum albumin. The equilibrium is shifted toward the dissociated form by mannitol 1-phosphate, resulting in a mannitol 1-phosphate induced inhibition. Mannitol does not affect the association state of the enzyme. Both mannitol and mannitol 1-phosphate also act as classical substrate inhibitors. The apparent K_i of each compound, however, is approximately equal to its apparent K_m, suggesting that mannitol and mannitol 1-phosphate bind at the same site on EII^{mtl}. Due to strong inhibition provided by mannitol and mannitol 1-phosphate in the exchange reaction, the kinetics of this reaction cannot be used to determine whether the reaction proceeds via a ping-pong or an ordered reaction mechanism.

Mannitol-specific enzyme II (EII^{mtl})[1] from the *Escherichia coli* phosphoenolpyruvate-dependent phosphotransferase system catalyzes both the transport and concomitant phosphorylation of mannitol at the expense of PEP with the help of two intracellular proteins, HPr and EI (see Scheme I). This

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[1] Abbreviations: PEP, phosphoenolpyruvate; EII^{mtl}, mannitol-specific enzyme II; EII^{Glc}, glucose-specific enzyme II; DTT, dithiothreitol; PTS, phosphoenolpyruvate-dependent sugar phosphotransferase system; BSA, bovine serum albumin; mtl, mannitol; mtl-1-P, mannitol 1-phosphate; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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