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Dissociation equilibria of the tryptophan synthase $\alpha_2\beta_2$ complex in saline buffer and guanidine isothiocyanate, as studied by sedimentation equilibrium

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Abstract

The dissociation equilibria of *Salmonella typhimurium* tryptophan synthase $\alpha_2\beta_2$ complex were studied via centrifugation of the complex to sedimentation equilibrium in neutral saline buffers containing 0 to 137 mM guanidine isothiocyanate (GuSCN). The resulting concentration gradients were analyzed in the context of an equilibrium model for sequential dissociation of two α subunits from a stable β_2 subunit. Under the conditions of these experiments, the first dissociation constant alone could be evaluated at GuSCN concentrations $\leq$ 100 mM, and the second dissociation constant alone could be evaluated at GuSCN = 137 mM. At intermediate GuSCN, both dissociation constants were sufficiently well defined to rule out the presence of a large equilibrium cooperative effect in the stepwise dissociation of the α subunits. Published by Elsevier Science B.V.

Keywords: Tryptophan synthase; Dissociation equilibria; Sedimentation equilibrium

1. Introduction

Tryptophan synthase [1] (EC 4.2.1.20) is a multisubunit enzyme composed of two α subunits (MW ca. 28,700) and one dimeric, tightly associated β_2 subunit (MW ca. 86,000) [1,2]. According to the 2.5 Å crystal structure of *Salmonella typhimurium* TS $\alpha_2\beta_2$ complex [3], the subunits are arranged in a quasilinear $\alpha\beta\beta\alpha$ order. Biochemical studies [4–6] have established that the enzyme is stabilized by the cofactor pyridoxal phosphate (2 moles/mole enzyme) and the substrate L-serine. Prior studies have indicated that upon dilution, the α subunits reversibly dissociate from the β_2 moiety, but there exists some uncertainty as to whether the two α subunits dissociate independently or cooperatively. On the basis of titration of enzymatic activity measured in the presence and absence of pyridoxal phosphate, Creighton and Yanofsky [4] concluded that the

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[1] Abbreviations used: TS, tryptophan synthase; PLP, pyridoxal phosphate; GuSCN, guanidine isothiocyanate; Bicine, *N,N*-bis(2-hydroxyethyl)glycine.

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two α subunits dissociated independently in *Escherichia coli* TS. Recently Banik et al. [6] concluded from similar experiments carried out on *S. typhimurium* TS that the dissociation of the second α subunit was inhibited by the dissociation of the first in the presence of PLP, but not in the absence of PLP. Negatively cooperative dissociation of α subunits was also suggested by Lane et al. [5] on the basis of their kinetic studies of the *E. coli* enzyme. In contrast, the results of equilibrium dialysis of mixtures of α and β subunits in the absence of pyridoxal phosphate suggest that dissociation of α subunits is positively cooperative [7]. Since previous studies of this phenomenon have yielded qualitatively different estimates of equilibrium dissociation constants as well as the extent of cooperativity in dissociation, it was decided to reexamine the dissociation equilibria of TS using the rigorous thermodynamic technique of sedimentation equilibrium.

It was found that in the neutral buffer employed here, our instrumental technique was insufficiently sensitive to permit us to evaluate reliably the equilibrium constant for dissociation of the second α subunit. However, an earlier study [8] had reported that chaotropic reagents promote subunit dissociation. We found that addition of the chaotropic reagent guanidine thiocyanate—at concentrations well below those at which large unfolding and denaturation effects are observed—enhances dissociation of α subunits to the point where a significant abundance of the fully dissociated β_2 species could be observed at accessible concentrations, permitting us to evaluate equilibrium dissociation constants for both α subunits.

2. Materials

S. typhimurium tryptophan synthase $\alpha_2\beta_2$ complex and isolated β_2 and α subunits were prepared as previously described in Refs. [9,10]. These were homogeneous as defined by polyacrylamide gel electrophoresis and size exclusion chromatography. Stock solutions were stored at $-20°$. Partial specific volumes, extinction coefficients and molecular weights for the $\alpha_2\beta_2$ complex and isolated α and β_2 subunits were taken from Adachi et al. [11]. Corresponding values for the partially dissociated species $\alpha\beta_2$ were calculated from subunit values assuming additivity. Standard neutral buffer, pH 7.8, contained 1 mM NaBicine, 1 mM NaEDTA, 100 mM NaCl, 1 mM dithiothreitol, and 20 μM pyridoxal phosphate. All reagents utilized were commercial-reagent grade, used without further purification. The density of buffer was measured pycnometrically at 20°C in the absence (1.003 g/cm^3) and presence of 100 mM GuSCN (1.011 g/cm^3), and it was assumed that density was linear in GuSCN concentration.

3. Methods

3.1. Sedimentation equilibrium

Stock enzyme solutions were thawed and diluted 1:10–1:60 into standard neutral buffer and dialysed overnight at 5°C. Three 12-mm double sector cells, each containing a 3-mm column height of sample solution (sample) and buffer (reference), layered over FC-47 fluorocarbon, were centrifuged simultaneously in an An Ti-60 rotor. Exact values of loading (plateau) absorbance were determined from initial scans taken just after speed had been reached. Loading absorbances were generally in the range 0.15–0.8 OD units at 280 nm. Runs were designed so that each successive cell of the three had approximately 1/2 to 1/3 the loading concentration of the cell which preceded it. Rotor speeds of between 5000 and 9000 rpm were employed, yielding equilibrium data that were reproducible between different runs of the same sample. Radial scans at 280 nm were taken every 6 or 8 h and equilibrium was judged to have been attained when subtracting one scan from a preceding scan yielded a flat line with amplitude indistinguishable from zero to within experimental uncertainty. This normally required from 32 to 48 h of sedimentation. Routinely, traces at 415 nm were also taken at the beginning and through the course of the run so as to monitor TS-bound PLP for anomalous behavior.

3.2. Analysis of data and determination of model parameter values

In order to establish that a system exists at sedimentation equilibrium and at chemical equilibrium, data (absorbance as a function of radial position)

were collected from multiple samples under each set of solution conditions at different rotor speeds and loading concentrations, and modeled globally to obtain a single best-fit set of the model parameters described in Sections 3.4 and 3.5. Global modeling reduces the possibility that nonequilibrium data may be fortuitously fit by an inappropriate equilibrium model with incorrect parameter values. Minimization of χ^2 was carried out using the Nelder–Mead modified simplex method [12]. The best fit of a model was judged to be in agreement with experimental data to within the precision of experimental error, with a confidence level of 95%, if the random probability of χ^2 being as large as that obtained from the best fit exceeded 0.05.

3.3. Determination of parameter uncertainty

The uncertainty in the value of the best-fit parameters was explored by the method of constrained least-squares fitting [13]. The value of a given parameter was constrained to a fixed value and the remaining parameters were allowed to vary in order to achieve a best fit. The cumulative distribution of squared residuals obtained from a single constrained fit is compared to that obtained from the unconstrained best fit and the value of the Kolmogorov–Smirnov D statistic calculated [12]. [2] The 95% confidence limits for a given parameter value correspond to the bounds of the range of the constrained parameter for which the probability of the null hypothesis: $P(D > D_{calc}) \geq 0.05$.

3.4. Models

3.4.1. Dissociation of TS

Preliminary sedimentation equilibrium experiments (data not shown) established that β_2 does not dissociate into monomeric β subunits under the conditions of this study at GuSCN concentrations ≤ 100 mM in the presence of 20 μM PLP. Thus the data are analyzed in the context of the following stepwise dissociation model:

$$\alpha_2\beta_2 \rightleftarrows \alpha + \alpha\beta_2 \quad \text{(I)}$$

$$\alpha\beta_2 \rightleftarrows \alpha + \beta_2 \quad \text{(II)}$$

We define the macroscopic dissociation constants

$$K_1 = \frac{c_\alpha c_{\alpha\beta_2}}{c_{\alpha_2\beta_2}} \quad (1)$$

$$K_2 = \frac{c_\alpha c_{\beta_2}}{c_{\alpha\beta_2}} \quad (2)$$

where concentrations are given in molar units. Because the arrangement of the two identical α subunits around the β_2 dimer is symmetric [3], we may also define an intrinsic site dissociation constant k and a cooperativity parameter γ that are related to the stepwise dissociation constants by [14]

$$K_1 = 2k_d \quad (3)$$

$$K_2 = \gamma k_d/2 \quad (4)$$

When γ is greater than unity, the second dissociation step is facilitated by the first, and when γ is less than unity, the second dissociation step is inhibited by the first.

3.4.2. Sedimentation equilibrium

At sedimentation equilibrium each individual species is presumed to sediment in accordance with the well known ideal sedimentation equilibrium equation [15]

$$c_{ij}(r) = c_{ij}(r_{ref}) \exp\left[\frac{M_{ij}(1-\bar{v}_{ij}\rho)\omega^2}{2RT}(r^2 - r_{ref}^2)\right] \quad (5)$$

where $c_{ij}(r)$ is the concentration of the species $\alpha_i\beta_j$ at radial position r, r_{ref} is an arbitrarily selected reference position, M_{ij} and $\bar{v}_{ij}$ are the molar mass and partial specific volume, respectively, of $\alpha_i\beta_j$, ρ is the solvent density, ω is the angular velocity of the rotor, R is the molar gas constant, and T is the absolute temperature. It is assumed that

$$M_{ij}(1-\bar{v}_{ij}\rho) = iM_\alpha(1-\bar{v}_\alpha\rho) + jM_\beta(1-\bar{v}_\beta\rho) \quad (6)$$

Eq. (6) is a valid approximation in the context of sedimentation equilibrium experiments carried out

[2] The Kolmogorov–Smirnov D statistic was selected as a measure of significance rather than the f or χ^2 statistics since no assumption of normal distribution of error is required. See section 13.5 of Ref. [12].

under the conditions of the present study, i.e., in short solution columns at low to moderate rotor velocities [14].

The absorbance of the solution at any radial position is assumed to be additive in the absorbances of the individual species:

$$A_\lambda(r) = \sum_{i,j} A_{ij,\lambda}(r) = \sum_{i,j} \epsilon_{ij,\lambda} c_{ij}(r) \quad (7)$$

and $\epsilon_{ij,\lambda}$, the molar extinction coefficient of $\alpha_i \beta_j$ at wavelength λ (corrected for light path length 1.2 cm) is assumed to be additive in the molar extinction coefficients of the individual subunits:

$$\epsilon_{ij,\lambda} = i\epsilon_{\alpha,\lambda} + j\epsilon_{\beta,\lambda} \quad (8)$$

The absorbance change at 280 nm measured upon mixing of α and β_2 subunits of TS does not exceed 2% of total absorbance [16], justifying the use of Eq. (8) at this wavelength.

3.5. Combined dissociation and sedimentation equilibria

The total (molar) concentrations of α and β subunits at a given radial position are

$$\alpha_{TOT}(r) = c_{10}(r) + c_{12}(r) + 2c_{22}(r) \quad (9)$$

$$\beta_{TOT}(r) = 2[c_{02}(r) + c_{12}(r) + c_{22}(r)] \quad (10)$$

For a sector-shaped cell, the total concentrations of α and β subunits, averaged over the entire solution, are given by

$$\langle \alpha_{TOT} \rangle = \frac{\int_{r_{men}}^{r_{base}} \alpha_{TOT}(r) r dr}{\int_{r_{men}}^{r_{base}} r dr} \quad (11)$$

and

$$\langle \beta_{TOT} \rangle = \frac{\int_{r_{men}}^{r_{base}} \beta_{TOT}(r) r dr}{\int_{r_{men}}^{r_{base}} r dr} \quad (12)$$

where r_{men} and r_{base} denote the radial positions of the solution meniscus and base, respectively. Both $\langle \alpha_{TOT} \rangle$ and $\langle \beta_{TOT} \rangle$ are functions of the average concentration of holoenzyme, and hence the average absorbance of enzyme:

$$\langle \alpha_{TOT} \rangle = \langle \beta_{TOT} \rangle = 2\langle c_{TS} \rangle = 2\langle A_{TS,\lambda} \rangle / \epsilon_{TS,\lambda} \quad (13)$$

where $\epsilon_{TS,\lambda}$ $(= 2\epsilon_{\alpha,\lambda} + 2\epsilon_{\beta,\lambda})$ denotes the molar extinction coefficient of the holoenzyme at wavelength λ.

Eqs. (1)–(13) may be used to calculate $A_\lambda(r)$ as a function of M_α, M_β, $\bar{v}_\alpha$, $\bar{v}_\beta$, ρ, $\epsilon_{\alpha,\lambda}$, $\epsilon_{\beta,\lambda}$, r_{men}, r_{base}, K_1, K_2 and $\langle A_{TS,\lambda} \rangle$. For purposes of modeling data obtained on dissociating systems, all quantities except K_1, K_2 and $\langle A_{TS,\lambda} \rangle$ are determined independently. Hence these three parameters are the only undetermined variables that are varied to achieve a best fit of the model to the data. [3] If mass is conserved over the course of the centrifuge run, $\langle A_{TS,\lambda} \rangle$ will be equal to the loading absorbance of the cell. To the extent that mass is not conserved, or to the extent that data are averaged over less than the full length of the solution column, $\langle A_{TS,\lambda} \rangle$ will be less than the loading absorbance of the cell.

4. Results

4.1. Association states of isolated α and β subunits

Although the isolated β subunit of *E. coli* TS is reported to exist as a stable dimer in solution [4], we performed sedimentation equilibrium experiments to verify the integrity of the β subunit of *S. typhimurium* TS in our buffer system. Equilibrium gradients of isolated α and β subunits (data not shown) are well fit by Eq. (5) for a single species of molar mass 27,000 and 84,500 g, respectively. These values are slightly smaller than the molar masses calculated from the chemical composition of *S. typhimurium* subunits (around 28,700 and 86,000, respectively [1,2], but the difference may be attributed to small errors or species differences in previously reported values of $\bar{v}_\alpha$ and $\bar{v}_\beta$, which were obtained from *E. coli* [11].

[3] In the actual calculation, log K_1, log K_2 and $\langle A_{TS,\lambda} \rangle$ were used as the fitting parameters to ensure that best-fit values of K_1 and K_2 are ≥ 0. When simultaneously modeling data obtained from n cells containing different loading concentrations, the number of fitting parameters is $2 + n$.

4.2. Dissociation of TS in aqueous buffer

The $\alpha_2\beta_2$ complex was centrifuged to sedimentation equilibrium over a range of enzyme concentrations. Fig. 1 shows results of experiments carried out at three enzyme concentrations in standard neutral buffer at 5°C. Absorbance gradients calculated using Eqs. (1)–(13) together with best-fit values of log K_1 and log K_2 are plotted together with the data. This result is typical of those obtained in several independent experiments. In Fig. 2 the significance of the Kolmogorov–Smirnov D statistic is plotted as a function of the constrained values of the parameters log K_1 and log K_2, respectively, in experiments carried out at 5°C. According to this analysis, these data are consistent with the following parameter values, with 95% confidence limits: log $K_1 = -5.70(+0.1, -0.7)$, log $K_2 \leq -5.0$. While the value of log K_1 is reasonably well defined by the data, only an upper limit for the value of log K_2 and hence γ may be established. Essentially identical results were obtained from comparable data obtained from an experiment at 20°C (data not shown).

4.3. Dissociation of $\alpha_2\beta_2$ in buffer with added GuSCN

Sedimentation equilibrium experiments similar to those carried out in the absence of GuSCN were carried out in buffer containing 50 mM, 100 mM, 112 mM, 125 mM, 137 mM and 150 mM GuSCN. For each GuSCN concentration, the model described in Section 3.4 was globally fitted to three absorbance gradients measured at different loading concentrations of enzyme. The raw data are not shown (but may be obtained upon request) because agreement between calculated best-fit and observed absorbance gradients was comparable to that obtained in the

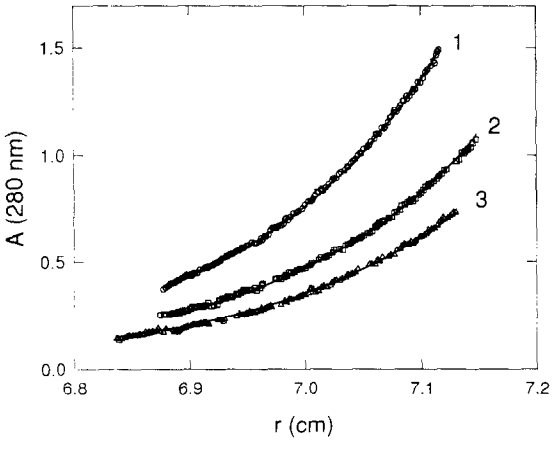

Fig. 1. 1. Equilibrium absorbance gradients of tryptophan synthase in standard neutral buffer, pH 7.8, at 5°C, 7000 rpm. Loading concentrations: (1) 1.3 mg/ml; (2) 0.83 mg/ml; (3) 0.50 mg/ml. Solid curves calculated for log $K_1 = -5.72$, log $K_2 = -8.20$.

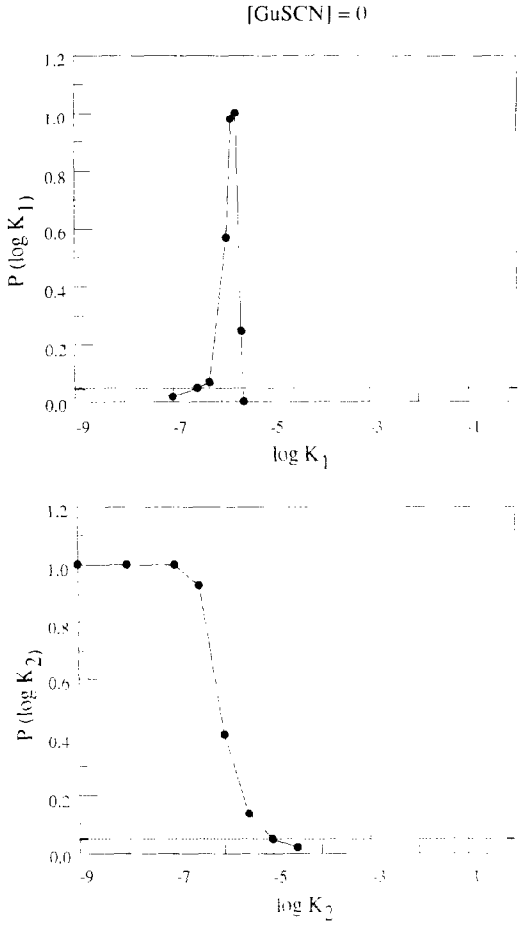

Fig. 2. Summary of results of analysis of constrained modeling of sedimentation equilibrium data obtained in the absence of added chaotropic agent, as described in Section 3. Abscissa is the value of the constrained parameter, and ordinate is the probability that by chance, the value of the Kolmogorov–Smirnov D statistic obtained from the cumulative distribution of two sets of points randomly selected from the same population will equal or exceed that calculated from the cumulative distributions of squared residuals obtained from the global best fit and the constrained best fit.

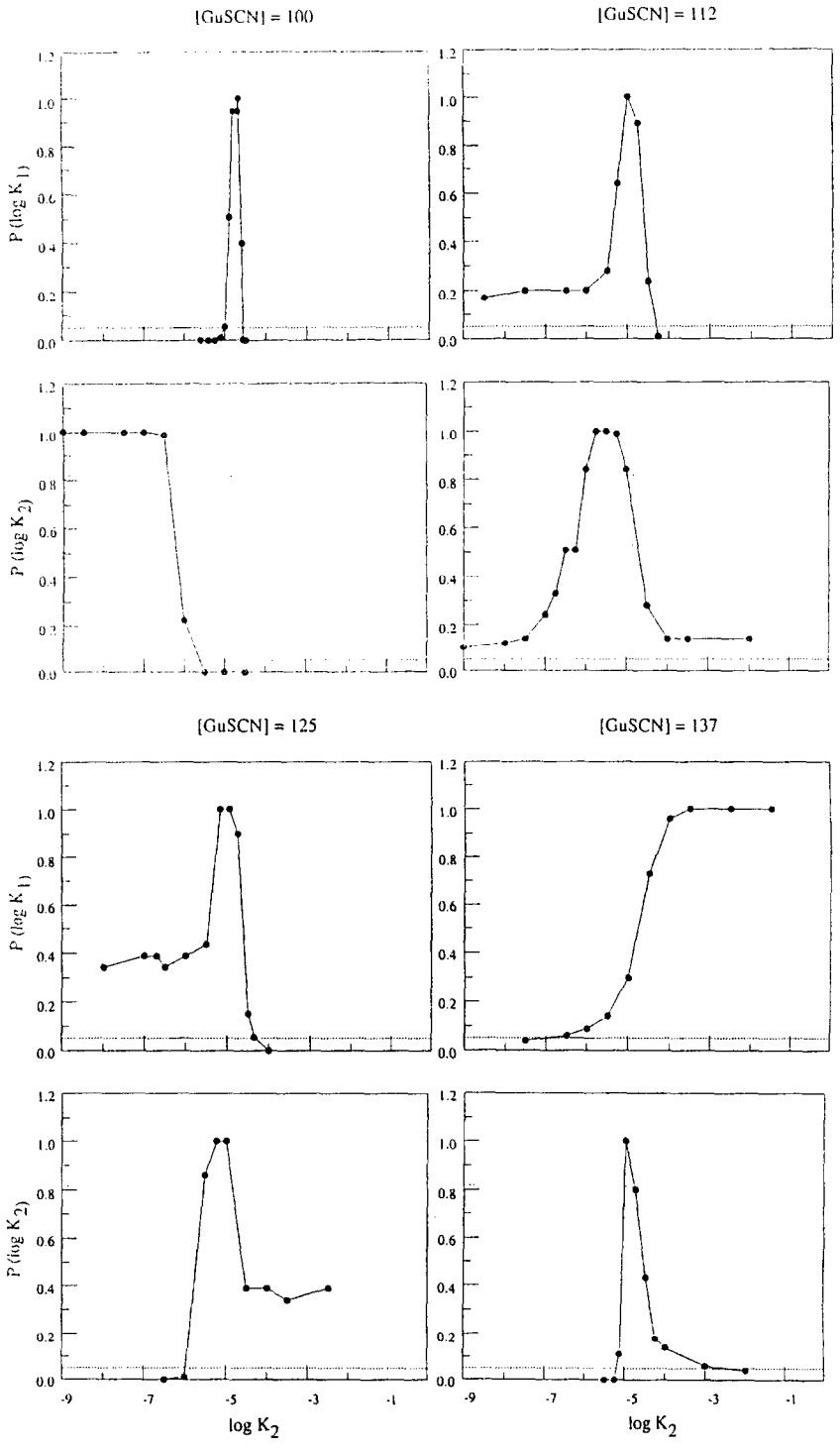

Fig. 3. Summary of results of analysis of constrained modeling of sedimentation equilibrium data obtained in the presence of variou concentrations of added chaotropic agent, as described in Sections 3 and 4.3. Axes of plots are as described in the caption to Fig. 2.

absence of GuSCN (cf. Fig. 1). Results obtained from multiple solutions in 150 mM GuSCN were not well modeled with uniform values of log K_1 and log K_2, suggesting the onset of processes (such as the dissociation of β_2 into individual β chains) that are not taken into account in our model. Uncertainty profiles for log K_1 and log K_2 obtained from modeling data at 100, 112, 125 and 137 mM GuSCN are presented in Fig. 3. At GuSCN concentrations exceeding 100 mM, log K_2 is resolved, and at 137 mM GuSCN only a lower limit for log K_1 can be established.

5. Discussion

5.1. Values of dissociation constants

Values of equilibrium dissociation constants for the apoenzyme (−PLP) and holoenzyme (+PLP) reported by prior investigators are summarized together with those reported here in Table 1. There are a number of possible reasons why the values reported for K_1 and K_2 vary substantially between the various studies.

(a) The study of Creighton and Yanofsky [4] was carried out on the enzyme from *E. coli* in the absence of PLP, the study of Lane et al. [5] was carried out on the same enzyme in the presence of PLP, that of Banik et al. [6] carried out on the enzyme from *S. typhimurium* in the presence and absence of PLP, and the current work carried out on the enzyme from *S. typhimurium* in the presence of PLP.

(b) In the studies of Creighton and Yanofsky [4] and Banik et al. [6], apparent equilibrium dissociation constants were derived from analysis of the dependence of the enzyme activity of a single subunit type (α or β) as a function of the relative concentrations of the two subunits in a mixture. These studies were carried out at 37°C, in the presence of saturating amounts of the substrate indole glycerol phosphate, which is known to stabilize the $\alpha_2\beta_2$ complex [4].

(c) Lane et al. [5] did not measure equilibrium dissociation constants, but calculated them as functions of postulated elementary rate constants obtained from analysis of stopped flow kinetic data interpreted in the context of a complex kinetic model. Such a procedure is susceptible to large propagation of error. In any event, although the experimental conditions of Lane et al. [5] are not so different from those of our own experiments, the dissociation constants calculated by them are so low that if they held even approximately under the conditions of our experiments, we should have observed that the enzyme sedimented virtually entirely as $\alpha_2\beta_2$ in the absence of GuSCN, which is not even approximately the case. However, this may be partly due to intrinsic differences between the *E. coli* and *S. typhimurium* enzymes.

Table 1
Equilibrium dissociation constants. Values reported by other authors have been converted into the constants defined by Eqs. (1) and (2)

K_1, K_2 (M)	Reference	Comments
5×10^{-7}, 1.25×10^{-7}	Creighton and Yanofsky [4]	37°C, pH 7.8, 0.1 M Cl^-, *E. coli*, in indole glycerol phosphate, (−PLP)
3×10^{-7}, 3×10^{-7}	Banik et al. [6]	37°C, pH 7.6, 50 mM NaBicine, *S. typhimurium*, in indole glycerol phosphate (−PLP)
2.2×10^{-5}, 1.4×10^{-5}	Bartholmes and Teusches [7]	22°C, pH 7.5, 0.1 M PO_4, *E. coli* (−PLP)
8×10^{-8}, $< 1 \times 10^{-8}$	Banik et al. [6]	37°C, pH 7.6, 50 mM NaBicine, *S. typhimurium*, in indole glycerol phosphate (+PLP)
1.5×10^{-7}, 3.8×10^{-8}		Same data as above, but fitwith noncooperative model (see Sections 3.4 and 5.3)
2×10^{-9}, 4×10^{-10}	Lane et al. [5]	25°C, pH 7.6, 0.1 M PO_4, *E. coli* (+PLP)
1×10^{-6}, 2.5×10^{-7}	Ogasahara et al. [17]	30°C, pH 7.0, 50 mM PO_4, *E. coli* (+PLP)
6.5×10^{-8}, 1.7×10^{-8}	Hiraga and Yutani [18]	25°C, pH 7.0, 50 mM PO_4, *E. coli* (+PLP)
2×10^{-7}, 5×10^{-8}	Hiraga and Yutani [18]	25°C, pH 7.0, 50 mM PO_4, *S. typhimurium* (+PLP)
2×10^{-6}, ND[a]	present work	5° and 20°C, pH 7.8, standard buffer, *S. typhimurium* (+PLP)

[a] Not determined.

(d) Two independent studies of $\alpha\beta$ interaction carried out by the same technique in the same laboratory, under nominally the same conditions [17,18] yielded values for the intrinsic dissociation constant k_d varying by more than a factor of 10. This suggests that the properties of the enzyme may vary significantly from preparation to preparation.

5.2. Uncertainty of parameter values

It was found that at GuSCN concentrations at or below 100 mM, only an upper limit for log K_2 may be determined, and at a GuSCN concentration of 137 mM, only a lower limit for log K_1 may be determined. The explanation for these findings is revealed by a calculation of the contribution of individual species to the total measured absorbance gradient. In Fig. 4 these contributions are plotted for three different GuSCN concentrations (0, 112 and 137 mM), using the parameter values given in the figure caption. For GuSCN = 0, log K_2 is the highest statistically acceptable value, and log K_1 is the corresponding best-fit value. For GuSCN = 137, log K_1 is the lowest statistically acceptable value, and log K_2 is the corresponding best-fit value. For GuSCN = 112, both log K_1 and log K_2 are best-fit values.

In the leftmost panel of Fig. 4, we see that in standard neutral buffer, under the conditions of our experiments, the species β_2 is present at concentrations so low that its contribution to the total absorbance gradient is comparable to the combined levels of instrumental noise and uncertainty in baseline position. Hence we cannot establish the value of the second dissociation equilibrium constant. In the rightmost panel of this figure, we see that under the same conditions, in the presence of 137 mM GuSCN, the fully undissociated species $\alpha_2\beta_2$ is present at a concentration so low that its contribution to the total absorbance gradient is comparable to the combined levels of instrumental noise and baseline uncertainty. Hence we cannot establish the value of the first dissociation equilibrium constant. In the intermediate case (112 mM GuSCN, middle panel) we see that all four species contribute significantly to the total absorbance, so that both dissociation constants may be evaluated.

5.3. Is dissociation of α subunits cooperative?

Our investigation was originally motivated by the question of whether the dissociation of tryptophan synthase is negatively cooperative, as had been suggested in some previous publications [5,6], or positively cooperative, as reported by Bartholmes and Teusches [7]. Eqs. (3) and (4) may be combined to yield

$$\log \gamma = \log 4 + \log K_2 - \log K_1 \qquad (14)$$

The results obtained in 112 mM and 125 mM GuSCN are consistent with values for log γ of 0.2 ± 0.7 and 0.6 ± 0.8, respectively. Although these values are rather uncertain due to the compounding of uncer-

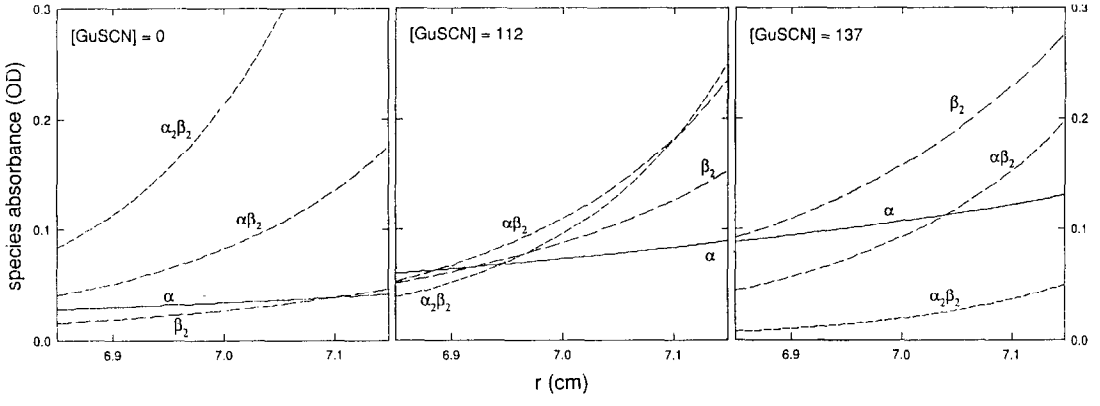

Fig. 4. Calculated absorbance gradients of individual species at 5°C, 7000 rpm, $A_{load} = 0.4$. Left panel: log $K_1 = -5.93$, log $K_2 = -6.0$ (or any smaller value). Center panel: log $K_1 = -5.11$, log $K_2 = -5.25$. Right panel: log $K_1 = -4.31$, log $K_2 = -4.75$.

tainty in the determination of both log K_1 and log K_2, they are seen to be inconsistent with strongly negative cooperativity (i.e., log $\gamma \leq -1$ and may possibly hint at some positive cooperativity in dissociation of this enzyme, although not as large as that reported by Bartholmes and Teusches [7] for the apoenzyme in the absence of PLP. We will not be able to determine whether this conclusion holds in the absence of chaotropic agent until a reliable technique for the quantitation of TS gradients at significantly lower total enzyme concentration is developed.

Our observation that the sequential dissociation of the two α subunits from the β_2 subunit is not markedly cooperative (at least in the presence of low concentrations of GuSCN) is in qualitative agreement with the conclusions of Creighton and Yanofsky [4]. This finding prompted us to return to the original reports of negatively and positively cooperative dissociation and reexamine the data.

According to Lane et al. [5], K_2 is approximately 4.8-fold smaller than K_1. According to Eqs. (3) and (4), for completely noncooperative two-step dissociation (i.e., $\gamma = 1$), K_2 would be four-fold smaller than K_1. We would argue that the values of K_1 and K_2 reported by Lane et al. [5] are likely to be sufficiently uncertain ($\pm > 20\%$) that their calculated value of K_1/K_2 is not statistically significantly different from 4.

Banik et al. [6] concluded that K_2 was at least eight-fold smaller than K_1 in the presence of PLP (but not in the absence of PLP) because they were able to fit the measured dependence of the specific catalytic activity of α subunit upon the concentration of added wild-type β_2 (measured in the presence of PLP) with a model in which K_2 was postulated to be vanishingly small. However, we have been able to fit the same data equally successfully with a model in which K_1/K_2 was constrained equal to four (see Table 1). Hence the data of Banik et al. [6] do not necessarily indicate the presence of negative cooperativity in the presence of PLP.

Bartholmes and Teusches [7] reported positively cooperative binding of the two α subunits to the apo-β_2 subunit (log $\gamma \approx 1.6$). While these experiments seem to reflect true equilibrium conditions, they were carried out in the absence of PLP, whereas our experiments were carried out in the presence of a large excess of PLP. However, addition of GuSCN may lead to partial dissociation of PLP, as suggested by a monotonic reduction of the absorbance ratio A_{410}/A_{280} of β_2 in the presence of increasing concentrations of GuSCN (data not shown). If such is the case, then the dissociation constants obtained via measurement of sedimentation equilibrium in the presence of moderate concentrations of GuSCN might reflect average properties of an ensemble of proteins that is less than fully saturated with PLP.

In summary, neither the present work nor any other quantitative study of which we are aware unequivocally indicates the presence of a significant degree of negative cooperativity in the sequential dissociation of the two α subunits from the β_2 subunit of tryptophan synthase. It follows that the free-energy changes accompanying dissociation of the first and second α subunits from the β_2 are roughly equal, and that the $\alpha\beta_2$ complex exhibits no special thermodynamic stability. It is stressed that this conclusion applies strictly to the case of dissociation equilibrium. We have no information as to the relative rates of dissociation of the two α subunits. Hence we cannot rule out the possibility that the intermediate $\alpha\beta_2$ is long-lived, i.e., stable in a kinetic sense, and hence present in some experimental situations at an abundance that is greater than would be predicted on the basis of equilibrium considerations alone.

A second objective of the present work was to explore the usefulness of a novel analysis of the dissociation equilibria of an unlabeled hetero-oligomeric protein using the analytical ultracentrifuge with absorption optics. The results show that the determination of multiple dissociation equilibrium constants is feasible only under favorable conditions, which in the present case were achieved by manipulation of solvent composition.

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