Differential Sedimentation Studies of Allosteric Interactions in *Pseudomonas* Tryptophan Oxygenase*

William N. Poillon† and Philip Feigelson‡

ABSTRACT: The technique of differential sedimentation was used to evaluate changes in the gross morphology of *Pseudomonas acidovorans* tryptophan oxygenase (L-tryptophan: oxygen oxidoreductase, EC 1.13.1.12) evoked by the binding of specific allosteric effectors. Two different conformational states of the protein molecule could be demonstrated, which were dependent on the status of the enzyme's allosteric site (saturated or unsaturated) and the valence state of its heme iron (trivalent or divalent). In the absence of effector molecules, the sedimentation coefficients of both the ferri- and ferroenzyme were identical (6.31 S) and designated "normal." This was the case for either valence species, whether the heme iron was unliganded or complexed by the appropriate ligand (carbon monoxide for the ferroenzyme and cyanide for the ferrienzyme). In the presence of allosteric effectors (tryptophan or α-methyltryptophan), however, the sedimentation coefficient of the ferroenzyme was increased to 6.73 S and designated "elevated," while that of the ferrienzyme was unaffected. Hence, an increased sedimentation rate of tryptophan oxygenase is observed when two conditions are satisfied: (1) saturation of its allosteric site and (2) maintenance of its heme iron in the divalent state; neither condition alone is sufficient to produce an elevated sedimentation coefficient. The change in hydrodynamic behavior observed when both conditions are met has been interpreted as indicating a contraction in effective molecular volume of approximately 18%. A model has been formulated which accounts for both the status of the allosteric site(s) and the heme iron valence state in determining the extent of conformational rearrangement during allosteric transitions of the tryptophan oxygenase molecule.

An intermediate molecular form exists in which the intrinsic conformational flexibility of the enzyme is only partially expressed. This appears to be the first reported instance in which the electronic configuration of a prosthetic group determines the alternate conformational isomers accompanying the allosteric transformations of an oligomeric protein.

The first step in the catabolism of L-tryptophan in *Pseudomonas acidovorans* is catalyzed by the enzyme tryptophan 2,3-dioxygenase (L-tryptophan:oxygen oxidoreductase, EC 1.13.1.12). Kinetic studies (Feigelson and Maeno, 1967) have shown that the rate of formation of the reaction product, L-formylkynurenine, is a sigmoidal function of the tryptophan concentration and that a profound diminution in the Michaelis constant for oxygen, $K_m^{O_2}$, is observed at saturating levels of tryptophan. A similarly increased apparent affinity for oxygen is observed in the presence of α-methyltryptophan, a substrate analog which, at the concentrations employed, is neither a substrate nor an inhibitor of the enzymatic activity. Furthermore, this substrate analog converts the sigmoidal tryptophan saturation curve to a hyperbolic one. A more recent study (Forman and Feigelson, 1971) presents evidence for the obligatory binding of tryptophan prior to oxygen at the enzyme's catalytic center.

The intrinsic catalytic potential of this enzyme is therefore subject to a type of regulation at the molecular level which may be designated as "allosteric" (Monod *et al.*, 1965; Koshland *et al.*, 1966). In this case, tryptophan binds to both the catalytic and allosteric sites, whereas α-methyltryptophan binds only to the allosteric or regulatory site(s) (Feigelson and Maeno, 1967).

In an attempt to account for the structural basis of these alterations in molecular architecture, the technique of differential sedimentation was employed. This method was first proposed by Richards and Schachman (1959), who showed the utility of interferometric techniques for the measurement of small differences in sedimentation coefficient. Later developments (Schachman, 1963; Lamers *et al.*, 1963) showed that the absorption system of the analytical ultracentrifuge could be used for such measurements as well. More recently, Schumaker and Adams (1968) have successfully used the schlieren optical system to measure extremely small changes (±0.016 S) in the sedimentation coefficient of rabbit IgG-immunoglobulin. Considerable enhancement of the precision in measuring absolute sedimentation coefficients is afforded by the simple expedient of performing sedimentation velocity analyses on two or more samples simultaneously in the same rotor. Accordingly, a series of sedimentation velocity experiments were undertaken in which the sedimentation coefficients of oxidized (Fe^{3+}) and reduced (Fe^{2+}) tryptophan oxygenase, either in the unliganded form or complexed, were measured in the presence of substrate and/or effector.

In order to distinguish between effects related to binding at the enzyme's allosteric site from those related to binding at its catalytic site, the substrate analogs α-methyltryptophan and 5-fluorotryptophan were used. The former has been shown, at the concentrations herein employed, to be ineffective as either substrate or inhibitor and to bind exclusively at the allosteric site (Feigelson and Maeno, 1967), while the latter is a competitive inhibitor of the enzyme's activity and presumably binds only at the catalytic site (K. Koike and

* From the Department of Biochemistry and the Institute of Cancer Research, College of Physicians and Surgeons, Columbia University, New York, New York 10032. *Received August 3, 1970.* This research was supported in part by U. S. Public Health Service Grants CA-02332 and CRTY-05011.

† Present address: Department of Biochemistry and Parkinson's Disease Research Center, College of Physicians and Surgeons, Columbia University, New York, N. Y. 10032.

‡ Career Investigator of the Health Research Council of the City of New York (I-104); to whom to address correspondence.

P. Feigelson, 1969, unpublished data). The use of tryptophan permitted evaluation of the effect of simultaneous saturation of both allosteric and catalytic sites on the enzyme's sedimentation rate.

The results obtained indicate that an increased sedimentation coefficient, corresponding to a contraction in molecular volume of about 18%, occurs when both of two conditions are satisfied: (1) saturation of the allosteric site by either tryptophan or α-methyltryptophan and (2) occurrence of the heme iron in the divalent state. Neither condition alone is sufficient for the manifestation of the elevation in sedimentation coefficient observed when both conditions are met. The structural basis for this behavior can be accommodated by a three-state model in which the valence state of the heme iron determines the extent of molecular rearrangement consequent to the binding of allosteric effectors.

Experimental Section

Materials. The enzyme was purified to a state of homogeneity from substrate-induced cultures of *Pseudomonas acidovorans* (ATC 11299) as described in a recent communication (Poillon *et al.*, 1969). Homogeneity was verified by analytical disc gel electrophoresis at pH 8.9 (Davis, 1964). Assays for enzymatic activity and protein content were performed after exhaustive dialysis against 0.1 M sodium phosphate buffer (pH 7). Tryptophan oxygenase activity was measured as described previously (Feigelson *et al.*, 1965) and protein concentration was determined spectrophotometrically at 405 mμ, using the value of 1.88 for the specific absorbance, $A_{1\,cm}^{0.1\%}$ (Poillon *et al.*, 1969). Each preparation was assessed to be >95% holo enzyme when exogenous hematin was omitted from the standard assay system.

The substrate, L-tryptophan, was obtained from Pierce Chemical Co., while the substrate analogs 5-fluorotryptophan and α-methyltryptophan were products of Nutritional Biochemicals Corp. and Regis Chemical Co., respectively. The Matheson Co. was the source of carbon monoxide and prepurified nitrogen.

Sedimentation Velocity Measurements. Sedimentation experiments were performed in a Spinco Model E analytical ultracentrifuge equipped with electronic speed control and regulated temperature indicator control units. The use of the split-beam photoelectric scanner, in conjunction with a multiplexer accessory, permitted the use of very dilute solutions of the enzyme (0.15–0.24 mg/ml) and the simultaneous monitoring of as many as three different samples in a single experiment. In this manner, errors in controlling rotor speed and temperature for separate experiments were eliminated and the precision in measuring relative differences in sedimentation rate was greatly enhanced. All experiments were performed in 12-mm double-sector cells with a four-hole An-F rotor. Sedimenting boundaries were monitored at the wavelength of the Soret absorption maximum appropriate for the species under inspection during a particular run. In order to ensure maximum stability, all experiments were performed at 4°, unless otherwise noted. Sedimentation coefficients were calculated from the slopes of the linear plots (fitted by the method of least squares) of the logarithm of boundary position *vs.* time and were corrected to values in a solvent with the viscosity and density of water at 20°. The concentration dependence of $s_{20,w}$ was considered negligible since all runs were performed at approximately the same low protein concentration (1.2–2.0 μM).

Technical Details of Individual Runs. Experiments were of three main types: (1) native enzyme (Fe^{3+}), in which the sixth coordination position of the ferriheme was either unoccupied or liganded to cyanide; (2) chemically reduced enzyme (Fe^{2+}), in which the sixth coordination position of the ferroheme was either unoccupied or liganded to carbon monoxide; and (3) enzyme plus ascorbate, in which the valence state of the heme iron, (Fe^{2+}) or (Fe^{3+}), was dependent on which of three effectors (tryptophan, 5-fluorotryptophan, or α-methyltryptophan) was present. Within each category, the effect of the appropriate substrate or substrate analog, applied either separately or in combination, on the absolute sedimentation coefficient of the enzyme was evaluated. All experiments were composed directly in the ultracentrifuge cell.

Since the ferroheme species tends to revert to the ferric form under aerobic conditions (Maeno and Feigelson, 1968a), the following precautions were taken with the reduced enzyme to keep the system as anaerobic as possible during manipulation. Stock solutions of the enzyme, its dialysate buffer and the various effectors dissolved in dialysate buffer were evacuated and flushed with nitrogen just prior to use; both sectors of the ultracentrifuge cell were flushed with nitrogen as well. Cells were filled with 0.25-ml syringes, taking care to expel all air. After aliquots of the enzyme, buffer and effector were in place, the reductant solution (either dithionite or ascorbate) was added, the cell quickly capped, and the contents mixed by inversion. After insertion of the cell into the rotor, the assembly was placed into the rotor chamber, which was then evacuated by action of the diffusion pump. In this manner, contact with the external milieu was minimal. Experiments with the ferriheme species were technically simpler, since no precautions were necessary to guard against aeration. In those experiments requiring the ligands carbon monoxide or cyanide, in addition to the other components, a suitable aliquot of a solution of the appropriate ligand was included in the total volume of 0.4 ml allotted to the solution sector of each cell. For the carbon monoxide system, gaseous carbon monoxide was bubbled into a small volume (10 ml) of cold dialysate buffer until saturation (~1 mM) was achieved, while for the cyanide system, a 0.2 M solution of potassium cyanide in dialysate buffer was used.

After equilibration to 4° and acceleration to 52,000 rpm (generally no more than 30 min after initial mixing), successive recorder traces were made at 405 and 432 mμ (unliganded ferroheme), 420 mμ (carbon monoxide liganded ferroheme), or 419 mμ (cyanide-liganded ferriheme) so as to obtain the appropriate spectral ratios for evaluation of the species present. The extent of reduction and, where appropriate, complex formation, were assessed from either the absorbance ratio A_{432}/A_{405} (unliganded) or A_{420}/A_{405} (carbon monoxide liganded), using the reference values of 2.0 and 2.7 obtained from the published spectra of the unliganded ferroenzyme (Poillon *et al.*, 1969) and its carbonmonoxy derivative (Maeno and Feigelson, 1968a), respectively. For the cyanide derivative of the ferrienzyme, the absorbance ratio, $A_{419}/A_{405} = 1.5$ was used (Maeno and Feigelson, 1968b). From these ratios, it was possible to verify that transformation to the appropriate species was complete in each instance. Other conditions relevant to individual sedimentation experiments are cited in the legends to the appropriate figure.

Results

Studies on the Chemically Reduced Enzyme. In Figure 1 are shown representative scans from a sedimentation velocity

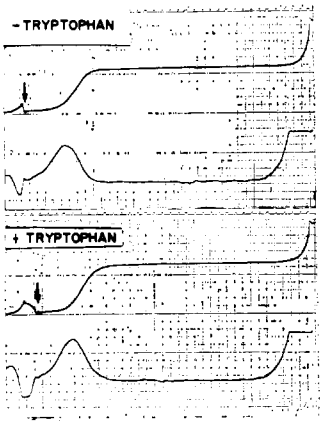


FIGURE 1: Photoelectric scanner traces from a sedimentation velocity experiment on chemically reduced tryptophan oxygenase (Fe^{2+}) in 0.1 M sodium phosphate (pH 7) at rotor speed 52,000 rpm, wavelength 405 mμ and temperature 4°. The upper and lower patterns represent the absence and presence of 3 mM tryptophan, respectively. The upper curve in each pattern corresponds to the absorbance as a function of distance (c *vs.* x) in the cell and the lower curve to its derivative (dc/dx *vs.* x). The scans depicted in the upper and lower patterns were recorded at 40 and 44 min after reaching speed, respectively, and the arrow denotes the position of the meniscus; direction of sedimentation is from left to right. Maximum pen deflection in the upper curves of each pattern corresponds to an absorbance of 0.2.

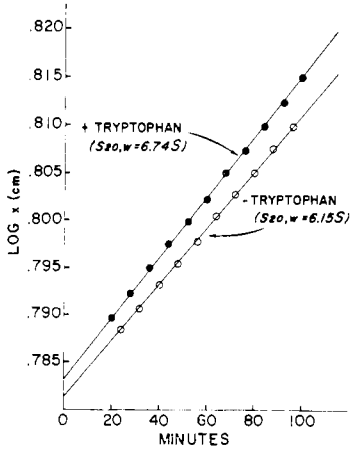


FIGURE 2: Effect of tryptophan on the sedimentation coefficient of chemically reduced tryptophan oxygenase (Fe^{2+}) in 0.1 M sodium phosphate (pH 7). Final concentrations of cell components were: tryptophan oxygenase, 1.2 μM; sodium dithionite, 1.8 mM; and, where appropriate, tryptophan, 3 mM. Both samples were sedimented simultaneously in an An-F rotor at 52,000 rpm and 4° and scans recorded at 8-min intervals at 405 mμ were used to determine sedimentation coefficients in the sample and reference cells. The ordinate represents the logarithm of the distance of the boundary (in centimeters) from the axis of rotation and the abscissa gives the time in minutes.

experiment on the unliganded form of the ferroheme enzyme, in the absence and presence of tryptophan. The reductant used was sodium dithionite and the spectral ratio A_{432}/A_{405} observed confirmed that the iron was completely divalent. These patterns are illustrative of the quality of the scans obtained in all experiments and from them the three principal features which characterize the enzyme solution during sedimentation are evident: (1) a uniformly flat plateau region; (2) a sharp, well-defined boundary; and (3) a transparent, or nearly so, supernatant region after the boundary has pulled away from the meniscus. When these three conditions are met within an individual run, the standard error of the log x *vs.* time plots was usually less than ±1%. In this case, the sedimentation coefficients, $s_{20,w}$, calculated were 6.33 and 6.62 S for the control system and that containing tryptophan, respectively.

As a test of reproducibility in measuring sedimentation coefficients between runs, the experiment described above was repeated under identical conditions; the plots of log x *vs.* time obtained are shown in Figure 2. It is evident that a significantly steeper slope is shown for the system containing tryptophan, than for the control system lacking substrate. The $s_{20,w}$ values derived from these data were 6.15 S (−tryptophan) and 6.74 S (+tryptophan). Hence, the precision in measuring sedimentation coefficients between these two runs was ±1.4% for the former system and ±0.9% for the latter. In general, an experimental precision of the order ±2% was encountered when the data of many different runs were evaluated (Table I). Most of this error can be attributed to variables such as fluctuation in rotor speed, thermal gradients within the rotor and inability to regulate and measure accurately the temperature of the ultracentrifuge cell.

The average sedimentation coefficients obtained from the data of Figures 1 and 2 indicate that in the presence of tryptophan, the sedimentation rate of the ferroenzyme is increased by about 7% relative to that of the control lacking tryptophan. Since the two solutions may be considered essentially equivalent with respect to ionic strength, viscosity, and density, this elevated sedimentation coefficient can be attributed to a specific effect of substrate binding.

Studies on the Carbon Monoxide Derivative of the Chemically Reduced Enzyme. Due to its enhanced stability, the ferrocarbonmonoxy complex was the preferred species for this series of experiments. Sedimentation coefficients were determined in the presence of the substrate analogs, 5-fluorotryptophan and α-methyltryptophan, either alone or in combination. For an experiment in which this carbon monoxide liganded species was sedimented in the presence of either 5-fluorotryptophan or α-methyltryptophan, the calculated sedimentation coefficients were 6.24 and 6.56 S, respectively. It is evident that only in the system containing α-methyltryptophan was the sedimentation coefficient significantly elevated relative to that of the unliganded ferroenzyme lacking effectors (*cf.* Figure 2, lower curve). The presence of 5-fluorotryptophan during sedimentation resulted in no such elevation and the sedimentation coefficient observed is identical with that observed in the absence of effectors.

In order to establish further the respective roles played by the allosteric *vs.* catalytic sites in this phenomenon, a similar experiment was performed in which solutions of carbon monoxide liganded ferroenzyme were sedimented separately in the presence of either 5-fluorotryptophan, tryptophan, or 5-fluorotryptophan plus α-methyltryptophan. In Figure 3 are shown the log x *vs.* time plots for these three systems. The $s_{20,w}$ values obtained were 6.40 S (+5-fluorotryptophan), 6.93 S (+tryptophan), and 6.82 S (+5-fluorotryptophan and α-methyltryptophan). It is evident that the combination of 5-fluorotryptophan plus α-methyltryptophan is equivalent in effect to that of tryptophan alone and that in both systems elevated sedimentation coefficients (6.82 and 6.93 S) are observed. In contrast, 5-fluorotryptophan alone is without effect and a normal value is observed for the sedimentation coefficient (6.40 S). It would appear, therefore, that an increased sedimentation rate occurs *only* when the

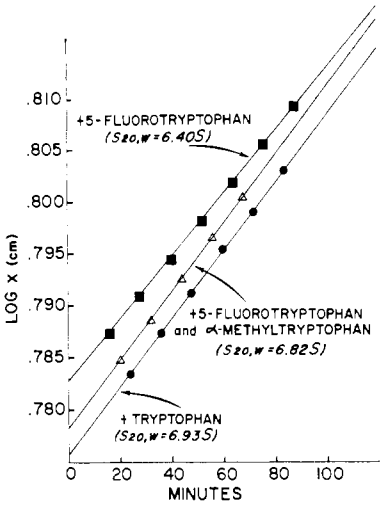


FIGURE 3: Effect of substrate and substrate analogs on the sedimentation coefficient of the carbon monoxide liganded derivative of chemically reduced tryptophan oxygenase (Fe^{2+}) in 0.1 M sodium phosphate (pH 7) at 4°. Final concentrations of cell components were: enzyme, 1.8 μM; sodium dithionite, 0.9 mM; carbon monoxide, 0.4 mM; and where appropriate, tryptophan, 3 mM; 5-fluorotryptophan, 5 mM; and α-methyltryptophan, 10 mM. Photoelectric scanner traces were recorded at 420 mμ; other conditions as cited in the legend to Figure 2.

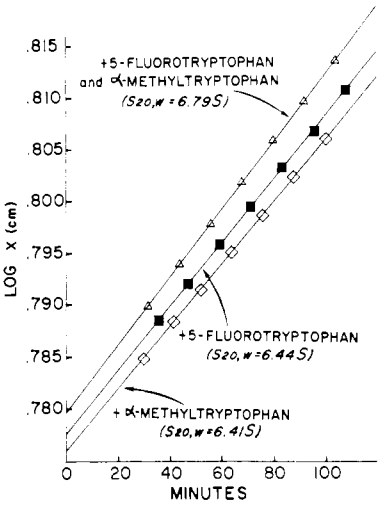


FIGURE 4: Effect of substrate analogs on the sedimentation coefficient of tryptophan oxygenase in the presence of sodium ascorbate. Cell components were composed in 0.1 M sodium phosphate buffer (pH 7) at 4° at final concentrations of: enzyme, 2 μM; sodium ascorbate, 2 mM; and, where appropriate, 5-fluorotryptophan, 5 mM; and α-methyltryptophan, 10 mM. Photoelectric scanner traces were recorded at 415 mμ, the isosbestic point between the oxidized (Fe^{3+}) and reduced (Fe^{2+}) forms of the enzyme. Other conditions are as cited in the legend to Figure 2.

allosteric site of the ferroenzyme is occupied by either tryptophan or α-methyltryptophan. Conversely, saturation of the catalytic site alone by 5-fluorotryptophan has no influence on the sedimentation rate.

Studies on the Enzymatically Reduced Enzyme. Experiments were undertaken to evaluate the conditions by which enzymatic reduction to the ferroheme species could be achieved in the ultracentrifuge cell. Accordingly, the sedimentation rate of tryptophan oxygenase (1.8 μM), initially in the oxidized form (Fe^{3+}) was examined in the presence of either 3 mM tryptophan, 2 mM sodium ascorbate, or both. From the spectral ratios, A_{432}/A_{405}, obtained for each system, it was evident that reduction to the ferroenzyme had occurred only when both tryptophan and ascorbate were present during sedimentation; neither tryptophan nor ascorbate alone sufficed to reduce the enzyme's heme iron to the divalent state. Such behavior could be predicted from the spectral and kinetic studies reported by Maeno and Feigelson (1967) and Tokuyama (1968).

Monitoring the sedimenting boundaries presented a special problem here, since in two of the three systems the oxidized form (Fe^{3+}) of the enzyme was present, while in the other, the reduced form (Fe^{2+}) occurred. As a compromise, then, sedimentation was followed in each system at 415 mμ, the isosbestic point between the Soret absorption peaks of the oxidized and reduced forms of the enzyme's heme iron (Poillon *et al.*, 1969). The boundary patterns obtained in the system containing only ascorbate were too noisy to permit an accurate estimation of sedimentation coefficient. The sedimenting boundaries of the system containing either tryptophan alone or tryptophan and ascorbate were quite stable, however, and values of 6.33 and 6.76 S were calculated for the respective sedimentation coefficients.

Additional experiments were performed in which tryptophan oxygenase, initially in the oxidized state (Fe^{3+}), was sedimented in the presence of either 5-fluorotryptophan, α-methyltryptophan, or both, each system including ascorbate as well. From the spectral ratios, A_{432}/A_{405}, observed for each system, it was evident that reduction to the ferroenzyme was achieved in either system which contained 5-fluorotryptophan and ascorbate, while in the system containing α-methyltryptophan and ascorbate, no such reduction occurred. From the log x *vs.* time plots shown in Figure 4 for each of these three systems, it was found that the combination of 5-fluorotryptophan and α-methyltryptophan results in an elevation of about 6% in the sedimentation coefficient, relative to the system containing either 5-fluorotryptophan (6.44 S) or α-methyltryptophan (6.41 S). Furthermore, the sedimentation coefficient observed for the enzymatically reduced enzyme in the presence of both 5-fluorotryptophan and α-methyltryptophan (6.79 S) is essentially identical with that previously cited (6.76 S) for the enzymatically reduced enzyme sedimented in the presence of tryptophan. In contrast, the sedimentation coefficients observed for either the 5-fluorotryptophan or α-methyltryptophan systems are in the range usually observed for the ferroenzyme in the absence of effectors. However, a fundamental distinction exists between these two systems, *i.e.*, in the former case (+5-fluorotryptophan) the heme iron is divalent and the allosteric site is vacant, while in the latter case (+α-methyltryptophan), the heme iron is trivalent and the allosteric site is occupied. Nevertheless, the sedimentation coefficients are indistinguishable and may be considered normal in both cases. Hence, the crucial importance of both divalent heme iron and saturation of the allosteric site for the manifestation of an elevated sedimentation coefficient is indicated. It will be recalled that both conditions are also met when the ferroenzyme is sedimented in the presence of tryptophan and an elevated sedimentation coefficient (6.76 S) is in fact observed.

Studies on the Cyanide Derivative of the Oxidized Enzyme. As an additional probe for evaluating the circumstances under which an elevated sedimentation coefficient may occur, experiments were performed with the cyanide-liganded

TABLE I: Summary of Normal *vs.* Elevated Sedimentation Coefficients Observed for Tryptophan Oxygenase under Various Conditions of Iron Valence State and Allosteric Site Saturation.

	Sedimentation Coeff ($s_{20,w}$) (S)[a]	
	Normal[b]	Elevated[c]
Mean value	6.31 ± 0.10[d]	6.73 ± 0.11[d]
Range	6.15–6.44	6.56–6.93
Number of determinations	16	16

[a] Sedimentation velocity experiments were performed at 52,000 rpm and 4°. [b] Observed when either the heme iron is divalent and the allosteric site is vacant or the heme iron is trivalent and the allosteric site is vacant or occupied by either tryptophan or α-methyltryptophan. [c] Observed *only* when the heme iron is divalent and the allosteric site is occupied by either tryptophan or α-methyltryptophan. [d] The plus and minus variations listed represent the standard deviation of the mean.

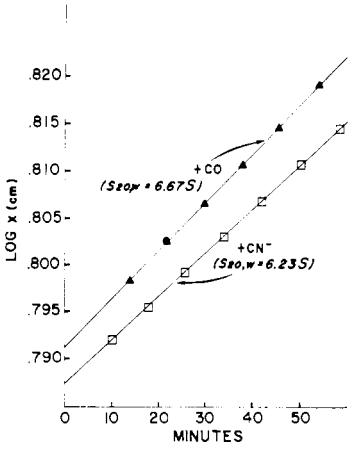


FIGURE 5: Effect of tryptophan on the sedimentation coefficient of the cyanide-liganded derivative of the oxidized (Fe^{3+}) and the carbonmonoxy-liganded derivative of chemically reduced (Fe^{2+}) tryptophan oxygenase in 0.1 M sodium phosphate (pH 7) at 20°. Final concentrations of cell components were: enzyme, 1.8 μM; tryptophan, 3 mM; and where appropriate, potassium cyanide, 1 mM; carbon monoxide, 0.4 mM; and sodium dithionite, 0.72 mM (reduced enzyme only). Photoelectric scanner traces were recorded at 420 mμ; other conditions as cited in the legend to Figure 2.

derivative of the oxidized (Fe^{3+}) enzyme. Solutions of ferritryptophan oxygenase (1.8 μM) were sedimented either in the absence or presence of 3 mM tryptophan; cyanide concentrations of 30 and 1 mM were employed in the former and latter cases, respectively. The spectral ratios, A_{419}/A_{405}, observed indicated that cyanocomplex formation was complete in each case. The sedimentation coefficient observed in the presence of tryptophan (6.15 S) was essentially identical with that of the control system (6.26 S) lacking substrate. Although the enzyme's allosteric site was saturated only in the former case, the sedimentation rate may be considered normal in both systems. It should be recalled that under similar conditions, with either the unliganded or the carbon monoxide liganded form of the chemically reduced enzyme (Fe^{2+}), an elevated sedimentation coefficient was observed in the presence of tryptophan. Hence, so long as the heme iron remains trivalent, saturation of the allosteric site by tryptophan is insufficient for the manifestiation of an elevated sedimentation coefficient.

Effect of Temperature on the Elevated vs. Normal Sedimentation Rates. Tryptophan oxygenase activity is routinely measured at room temperature (~25°), while the sedimentation experiments reported heretofore were performed at 4°. Therefore, in order to evaluate whether the well-defined conditions under which an elevated sedimentation coefficient may be observed was temperature sensitive, the following experiments were performed at 20°. Both the cyanide-liganded derivative of the oxidized enzyme (Fe^{3+}) and the carbon monoxide liganded derivative of the chemically reduced enzyme (Fe^{2+}) were separately sedimented in the presence of tryptophan. The log *x vs.* time plots obtained are shown in Figure 5. It can be seen that a significantly steeper slope is shown by the data for the carbon monoxide liganded derivative of the ferroenzyme, relative to that of the cyanide-liganded derivative of the ferrienzyme. The sedimentation coefficients calculated from these data were 6.67 and 6.23 S, respectively. Hence, an elevated sedimentation coefficient of about the same order of magnitude as that seen at 4° is found at 20° as well, so long as the requirements for divalent iron and allosteric site saturation are fulfilled. It would appear, therefore, that this phenomenon is independent of temperature, at least over the range 4–20°.

In Table I are summarized the data for the sedimentation coefficients observed for tryptophan oxygenase in the presence or absence of various effectors, as a function of the valence state of its heme iron. Where appropriate, substrate (tryptophan) or substrate analogs (5-fluorotryptophan or α-methyltryptophan) were present at sufficiently high concentrations (3, 5, and 10 mM, respectively) to ensure saturation of their respective stereospecific receptor sites on the enzyme. In addition, the valence state of the heme iron was maintained either as trivalent, the usual form for the purified native enzyme, or by suitable reduction, as the divalent form. Reduction to the latter state was achieved either chemically, with sodium dithionite or enzymatically, with sodium ascorbate and either tryptophan or 5-fluorotryptophan.

It is evident that the sedimentation coefficients observed could be unambiguously assigned to one of two categories, which for the sake of convenient notation are designated "normal" or "elevated." For all experiments in which the enzyme's heme iron was trivalent and its allosteric site was vacant or saturated with either tryptophan or α-methyltryptophan or in which its heme iron was divalent and its allosteric site was vacant, a normal sedimentation coefficient (6.31 ± 0.10 S) was observed. This value is in good agreement with that of 6.26 ± 0.16 S previously reported by Poillon *et al.* (1969) as the mean value for seven different preparations of the oxidized (Fe^{3+}) enzyme in the absence of effectors. On the other hand, for all experiments in which the enzyme's heme iron was maintained in the divalent state and its allosteric site was saturated with the appropriate effector, an elevated sedimentation coefficient (6.73 ± 0.11 S) was observed, which is 6.6% higher than the normal value. These results clearly show that the valence state of the heme iron determines which species will be observed during sedimentation of the effector-saturated enzyme. The more rapidly sedimenting form is observed only when the iron is divalent. Thus, separate and distinct roles are played by the iron

valence state, on the one hand, and the status of the allosteric site, on the other, in producing a detectable alteration in the hydrodynamic properties of tryptophan oxygenase.

It should be noted also that it is solely the valence state of the heme iron which determines whether a normal or elevated sedimentation coefficient is manifested under the condition of allosteric site saturation; the spin state configuration of the iron has no bearing on this phenomenon. Thus, the $s_{20,w}$ values observed for the unliganded ferroenzyme, where a high-spin configuration predominates (Poillon *et al.*, 1969) and for its carbonmonoxy-liganded derivative, in which a low-spin configuration presumably occurs (Peisach *et al.*, 1968) are both elevated. Conversely, the $s_{20,w}$ values observed for the unliganded ferrienzyme, where a high-spin configuration predominates (Poillon *et al.*, 1969) and for its cyanide-liganded derivative, in which a low-spin configuration exists, are both normal.

Discussion

These sedimentation velocity studies indicate that the interaction of ferrotryptophan oxygenase with specific effectors which saturate its allosteric sites produces a species in which the sedimentation coefficient is elevated by 6.6%. Since the magnitude of this change is too small to be attributable to a change in the state of aggregation of the tetrameric tryptophan oxygenase molecule, it must reflect instead a reversible change in molecular conformation. Since the increase in mass on binding of effector (tryptophan or α-methyltryptophan) to one or more allosteric sites on the protein is negligible (0.18%/mole of effector bound), this change in sedimentation coefficient presumably reflects a decrease in the frictional coefficient ratio, f/f_0, of the sedimenting hydrodynamic particle. In terms of the gross morphology of oligomeric, globular proteins, such a decreased frictional ratio results from an alteration of either the shape term (axial ratio) or the relative size term (volume ratio of the equivalent hyd. ed to anhydrous spheres) which together comprise this hydrodynamic parameter (Schumaker, 1968). However, when the alteration in sedimentation rate caused by a low molecular weight effector is small, the change in axial ratio of the hydrated ellipsoid of revolution by which most globular proteins may be represented, is considered negligible and the conformational change may be interpreted solely in terms of the effective hydrodynamic volume. In this case, then, since the change in sedimentation coefficient resulting from effector binding is positive, a molecular contraction is indicated, which produces a sedimenting particle of more compact conformation.

The quantitative expression of such sedimentation rate changes has been formulated in a recent theoretical paper by Schumaker (1968). In his treatment, the observed alteration in sedimentation coefficient is related to the fractional change in molecular frictional ratio by the expression, $\delta q/q = (K/(1 + \delta s/s)) - 1$, where q corresponds to the frictional ratio (f/f_0), s to the sedimentation coefficient, and K to a constant whose value is close to unity. The symbols $\delta q = q' - q$ and $\delta s = s' - s$ refer to the differential frictional ratio and sedimentation coefficient, respectively, before (unprimed) and after (primed) the addition of effector molecule. Since shape alterations may be considered negligible when δs is small, the quantity $\delta q/q$ may be interpreted in terms of the fractional expansion or contraction of the protein consequent to the binding of effector. (For a detailed presentation of the various assumptions and approximations implicit in this treatment, the reader is referred to the original paper (Schumaker, 1968).)

In this case, substitution of the experimentally determined values of 6.73 and 6.31 S for s' and s, respectively, into the above expression yields a value of -6.2% for the parameter $\delta q/q$, which in turn corresponds to a contraction in effective hydrodynamic volume of about 18% (*i.e.*, $\delta q/q$ is approximately equal to one-third of the fractional contraction of the protein). The absolute values of q and q' calculated from the appropriate sedimentation coefficient and a molecular weight of 122,000 for the native enzyme (Poillon *et al.*, 1969) are 1.36 and 1.28, respectively. Thus, when the heme iron is divalent, the binding of effectors (tryptophan or α-methyltryptophan) to a stereospecific allosteric receptor site(s), which is topographically distinct from the catalytic site(s), results in a significant decrease in effective hydrodynamic volume of the tryptophan oxygenase molecule.

A few examples of behavior analogous to that documented here for tryptophan oxygenase are available. The X-ray data of Muirhead and Perutz (1963) indicate a decrease in molecular volume of about 8% upon oxygenation of hemoglobin. From this, it may be calculated that the sedimentation coefficient would increase by about 3% upon oxygenation. Similarly, De Phillips *et al.* (1969) have shown that oxygenated hemocyanin sediments 2.4% faster than the deoxygenated species. This would correspond to a decrease in molecular volume of 7.2% upon oxygenation. The most rigorous demonstration of a reversible conformational change in an oligomeric protein upon binding an allosteric effector may be found in the elegant study of Gerhart and Schachman (1968). These authors showed that in the presence of carbamyl phosphate and succinate, the sedimentation coefficient of *Escherichia coli* aspartate transcarbamylase decreased by about 4%. This corresponds to a fractional change in frictional ratio of +4.2% and an expansion of near 13% in molecular volume.

Since these changes are of the same order of magnitude as those observed here, it seems likely that the alteration of molecular volume detected for ferrotryptophan oxygenase on binding allosteric effectors is equivalent to, or at least related to, those cooperative interactions which mediate the allosteric regulation of enzymatic function and which are expressed kinetically by a sigmoidal substrate saturation curve for tryptophan and a drastically reduced Michaelis constant for oxygen in the presence of substrate (Feigelson and Maeno, 1967).

From the information currently available, it is not possible to distinguish between the two proposed models for allosteric transitions in oligomeric proteins, *i.e.*, those of Monod–Wyman–Changeux (1965) and Koshland–Nemethy–Filmer (1966). In the former model, an equilibrium between two alternate conformations is displaced in concerted fashion by effector binding, while in the latter model the displacement of such an equilibrium occurs in a sequential manner, whereby the binding of effector induces a conformational change only in the individual subunit to which it is bound. Formally then, the fundamental distinction between the two models is that only in the latter one do intermediate states exist in which the structural conformation of the individual subunits is determined by the degree of saturation with effector. An attempt has been made to accommodate qualitatively the findings presented here into a model which accounts for the allosteric transformations observed for tryptophan oxygenase. For this purpose, the various permutations of heme iron valence state and allosteric site status, as well as the corre-

TABLE II: Some Properties of the Conformational States of Tryptophan Oxygenase.

Valence State	Allosteric Site	$S_{20,w}$	Quaternary Structure Stability[a]	State
Fe^{3+}	Vacant	Normal	Normal	R
Fe^{3+}	Occupied	Normal	Enhanced	Incomplete T
Fe^{2+}	Vacant	Normal	—[b]	?
Fe^{2+}	Occupied	Elevated	—[b]	Complete T

[a] As determined by the sodium dodecyl sulfate:protein ratio (w/w) required to effect 50% disruption of tetrameric tryptophan oxygenase (Koike *et al.*, 1969, Figure 9). [b] Undetermined.

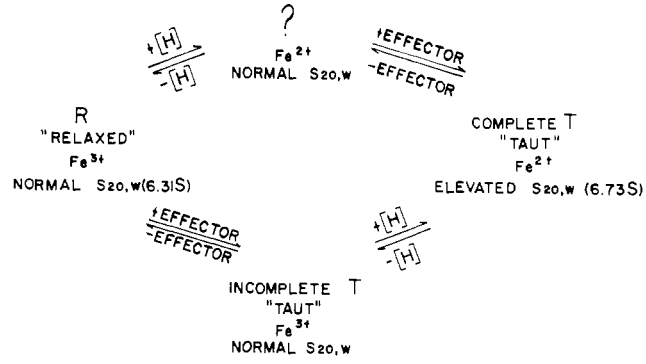


FIGURE 6: Schematic outline of the conformational states of tryptophan oxygenase as determined by the heme iron valence state and the status of the allosteric site(s). Reduction of the heme iron, denoted by the symbol [H], may be achieved either chemically (sodium dithionite) or enzymatically (sodium ascorbate + tryptophan or 5-fluorotryptophan). The status of the allosteric site is determined by the presence (+) or absence (−) of the effectors, α-methyltryptophan or tryptophan.

sponding sedimentation coefficients observed in each case are presented schematically in Figure 6. The protomeric form of tryptophan oxygenase is a tetramer of mass 122,000 daltons, to which is affixed one protoheme group. The regulation of enzymatic activity by specific allosteric site–catalytic site interactions is accommodated by structural alterations within this fundamental unit.

In the purified native enzyme (tryptophan free) (Poillon *et al.*, 1969), the heme iron occurs in the trivalent state and the sedimentation coefficient of 6.31 S observed is termed normal. This species is designated as R or "relaxed" in Figure 6. Saturation of the allosteric site of the ferrienzyme with the appropriate effector (tryptophan or α-methyltryptophan) converts the R form into a species designated as incomplete T, which sediments in the ultracentrifuge at a rate in the normal range. Nevertheless, evidence that a strengthening of the specific noncovalent bonds which stabilize the enzyme's quaternary structure occurs in this transition is afforded by the study of Koike *et al.* (1969) (Table II). These authors showed that when the anionic detergent sodium dodecyl sulfate was used to dissociate the native enzyme into its four constituent subunits, a considerably higher ratio of detergent to protein was required if the effector, α-methyltryptophan, was present with the disruptive agent. It would appear, therefore, that the "tightening" of internal structure evoked by the allosteric effector in this case is of insufficient magnitude to alter detectably the sedimentation coefficient. Subsequent conversion of the heme iron of the incomplete T state from trivalent to divalent by suitable reduction results in a species designated as T, or "taut," whose sedimentation coefficient of 6.73 S is appreciably elevated from the normal value of 6.31 S.

An alternative route to the complete T state is provided by the upper pathway of Figure 6. The initial reduction of the heme iron from trivalent to divalent results in a species whose sedimentation coefficient is in the normal range, but whose sensitivity to disruption by sodium dodecyl sulfate has not been evaluated, and whose molecular structure is therefore designated as "?" in Figure 6. Subsequent exposure of this species to the appropriate allosteric effector converts it into one whose sedimentation coefficient is in the elevated range, *i.e.*, transforms it to the complete T state. The properties of the various conformational isomers depicted in Figure 6 are summarized in Table II.

It is apparent, therefore, that a more profound modification of molecular structure occurs in the transition R $\rightleftharpoons$ complete T, than in the one R $\rightleftharpoons$ incomplete T and that energetically, the complete T state should be the most stable, *i.e.*, the species in which the intersubunit bonds are strongest. The necessity of imposing an intermediate state between the two conformational isomers in order to accommodate the findings reported here arises from the additional parameter which one does not encounter with a simple, unconjugated allosteric protein, *viz.*, the valence state of the heme iron. This appears to be the first documented instance in which the valence state of a prosthetic group plays a crucial role in regulating the conformational isomerization of an oligomeric protein molecule.[1] In a formal sense, then, the electron which converts the trivalent iron into the divalent state is as much an effector of the cooperative interactions between the allosteric and catalytic sites as is the substrate tryptophan or its analog, α-methyltryptophan. It would appear that an additional conformational constraint is imposed upon the tryptophan oxygenase molecule when its heme iron is trivalent, which prevents the complete transition to its alternate form when small molecule effector is bound and instead the conversion is only partial. This constraint is apparently relieved when the iron is reduced to the divalent state and complete expression of the potential molecular rearrangement can occur upon binding of small molecule effector. These findings indicate that intermediate conformational states can occur in the allosteric transitions of the tryptophan oxygenase molecule.

References

Azzi, A., Fleischer, S., and Chance, B. (1969), *Biochem. Biophys. Res. Commun. 36*, 322.

Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.

De Phillips, H. A., Nickerson, K. W., Johnson, M., and Van Holde, K. E. (1969), *Biochemistry 8*, 3665.

Feigelson, P., Ishimura, Y., and Hayaishi, O. (1965), *Biochim. Biophys. Acta 96*, 283.

[1] Using the fluorescent probe, 8-anilino-1-naphthalenesulfonic acid, it has recently been shown (Azzi *et al.*, 1969) that a structural change occurs during the transition ferri- to ferrocytochrome *c* in a cytochrome *c*–phospholipid complex. Such behavior seems analogous to that reported here.

Feigelson, P., and Maeno, H. (1967), *Biochem. Biophys. Res. Commun. 28*, 289.
Forman, H., and Feigelson, P. (1971), *Biochemistry 10*, 760.
Gerhart, J. C., and Schachman, H. K. (1968), *Biochemistry 7*, 538.
Koike, K., Poillon, W. N., and Feigelson, P. (1969), *J. Biol. Chem. 244*, 3457.
Koshland, D., Nemethy, G., and Filmer, D. (1966), *Biochemistry 5*, 365.
Lamers, K., Putney, F., Steinberg, K. F., and Schachman, H. K. (1963), *Arch. Biochem. Biophys. 103*, 379.
Maeno, H., and Feigelson, P. (1967), *J. Biol. Chem. 242*, 596.
Maeno, H., and Feigelson, P. (1968a), *J. Biol. Chem. 243*, 301.
Maeno, H., and Feigelson, P. (1968b), *Biochemistry 7*, 968.
Monod, J., Wyman, J., and Changeux, J.-P. (1965), *J. Mol. Biol. 12*, 88.
Muirhead, H., and Perutz, M. F. (1963), *Nature (London) 199*, 633.
Peisach, J., Blumberg, W. E., Wittenberg, B. A., and Wittenberg, J. (1968), *J. Biol. Chem. 243*, 1871.
Poillon, W. N., Maeno, H., Koike, K., and Feigelson, P. (1969), *J. Biol. Chem. 244*, 3447.
Richards, E. G., and Schachman, H. K. (1959), *J. Phys. Chem. 63*, 1578.
Schachman, H. K. (1963), *Biochemistry 2*, 887.
Schumaker, V. (1968), *Biochemistry 7*, 3427.
Schumaker, V., and Adams, P. (1968), *Biochemistry 7*, 3422.
Tokuyama, K. (1968), *Biochim. Biophys. Acta 151*, 76.

Kinetic Evidence Indicating the Absence during Catalysis of an Unbound Ferroprotoporphyrin Form of Tryptophan Oxygenase*

Henry Jay Forman and Philip Feigelson†

ABSTRACT: A kinetic study of the mechanism of catalysis of tryptophan oxygenase using carbon monoxide as an inhibitor is presented. The study was carried out with the aid of α-methyltryptophan to saturate the allosteric site thereby allowing formulation of simplified rate equations and the generation of linear reciprocal kinetic plots. Various proposed mechanisms are evaluated; the data are incompatible with the existence during the steady-state reaction of any tryptophan-free form of ferrotryptophan oxygenase which is capable of binding either oxygen or carbon monoxide. The mechanism which is compatible with all the data involves an ordered addition of tryptophan to the enzyme followed by oxygen. As oxygen will not bind to trivalent heme, one possible explanation for this requirement is the reduction, during catalysis, of the enzyme-bound ferriprotoporphyrin IX moiety by tryptophan.

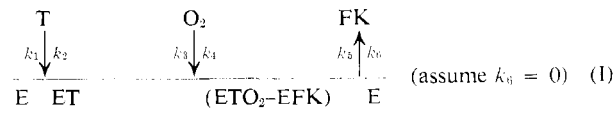
Tryptophan 2,3-dioxygenase (EC 1.13.1.12), a heme protein, catalyzes the reaction of the pyrrole moiety of tryptophan with oxygen resulting in the formation of formylkynurenine.

Maeno and Feigelson (1967) and Hayaishi (1969) have carried out studies which show that, in the absence of tryptophan, the trivalent and divalent forms of heme in tryptophan oxygenase are, respectively, unaffected and slowly autooxidized by oxygen. When dithionite reduction of the ferriheme moiety of the enzyme is performed the ferrotryptophan oxygenase obtained is capable of binding carbon monoxide in the absence of tryptophan. All trivalent forms of ferritryptophan oxygenase will not bind carbon monoxide. No form of the enzyme in its divalent state has been found as yet which will not bind CO. The valence state of the heme iron of the enzyme during various stages of catalysis and particularly the valence state to which it returns after releasing formylkynurenine before tryptophan is bound again has remained the most interesting and difficult problem concerning the catalytic mechanism of this dioxygenase. In this paper a new approach to the problem was applied which confirmed the order of binding of the substrates as tryptophan before oxygen, and supports the concept of fluctuation of the valence state of the heme iron during catalysis (as depicted in Figure 1A). The properties of enzyme which has been chemically reduced by dithionite were compared to those found for the enzyme during the steady state resulting in rejection of the former as a participating form in the catalytic cycle.

Kinetic Theory

In previous papers (Feigelson *et al.*, 1969; Koike *et al.*, 1969) the allosteric properties of tryptophan oxygenase were discussed. As shown in these papers, α-methyltryptophan reduces the complex behavior of the enzyme such that the simple mechanisms below need only be considered. The concentration of α-methyltryptophan herein employed (9.6×10^{-4} M) is such that it saturates the allosteric site and does not combine with the catalytic site. Thus, in these experiments considerations of allosterism are eliminated from further consideration.

Various mechanisms for the catalytic reaction of tryptophan oxygenase could be postulated without consideration of its valence state (eq I–III).



(assume $k_6 = 0$) (I)

* From the Institute of Cancer Research and the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York, New York 10032. *Received August 3, 1970.*

† Career Investigator I-104, Health Research Council of the City of New York; to whom to address correspondence.