THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 10, Issue of March 10, pp. 6956–6962, 2000

Printed in U.S.A.

Effect of pH and Monovalent Cations on the Formation of Quinonoid Intermediates of the Tryptophan Synthase $\alpha_2\beta_2$ Complex in Solution and in the Crystal*

(Received for publication, November 27, and in revised form, December 10, 1999)

Andrea Mozzarelli‡§¶, Alessio Peracchi‡, Barbara Rovegno‡, Giovanna Dalè‡, Gian Luigi Rossi‡, and Michael F. Dunn‖

From the ‡Institute of Biochemical Sciences and §National Institute for the Physics of Matter, University of Parma, 43100 Parma, Italy and the ‖Department of Biochemistry, University of California at Riverside, Riverside, California 92521

Quinonoid intermediates play a key role in the catalytic mechanism of pyridoxal 5′-phosphate-dependent enzymes. Whereas the structures of other pyridoxal 5′-phosphate-bound intermediates have been determined, the structure of a quinonoid species has not yet been reported. Here, we investigate factors controlling the accumulation and stability of quinonoids formed at the β-active site of tryptophan synthase both in solution and the crystal. The quinonoids were obtained by reacting the α-aminoacrylate Schiff base with different nucleophiles, focusing mainly on the substrate analogs indoline and β-mercaptoethanol. In solution, both monovalent cations (Cs^+ or Na^+) and alkaline pH increase the apparent affinity of indoline and favor accumulation of the indoline quinonoid. A similar pH dependence is observed when β-mercaptoethanol is used. As indoline and β-mercaptoethanol exhibit very distinct ionization properties, this finding suggests that nucleophile binding and quinonoid stability are controlled by some ionizable protein residue(s). In the crystal, alkaline pH favors formation of the indoline quinonoid as in solution, but the effect of cations is markedly different. In the absence of monovalent metal ions the quinonoid species accumulates substantially, whereas in the presence of sodium ions the accumulation is modest, unless α-subunit ligands are also present. α-Subunit ligands not only favor the formation of the intermediate, but also reduce significantly its decay rate. These findings define experimental conditions suitable for the stabilization of the quinonoid species in the crystal, a critical prerequisite for the determination of the three-dimensional structure of this intermediate.

The bacterial tryptophan synthase $\alpha_2\beta_2$ complex catalyzes the last two steps in the biosynthesis of L-tryptophan (1–4). Indole is formed from the cleavage of indole-3-glycerol phosphate in the α-active site and subsequently is channeled, via a hydrophobic tunnel, to the β-active site (5, 6) where it is combined with L-Ser to make L-Trp. The reaction at the β-active site depends on the cofactor pyridoxal 5′-phosphate and proceeds through the formation of several intermediates, which are characterized by distinct absorption properties (Scheme 1) (2, 7–10).

The α- and β-subunit activities are allosterically regulated (2–4, 6, 11–20) via the selective stabilization of α- and β-subunit conformations consisting of an "open," catalytically inactive state, and a "closed" catalytically active state (11, 12, 21, 22). The internal and the external aldimines in the β-active site are in open and partially open conformations, respectively, and do not send regulatory signals to the α-active site (4, 12–14). The α-aminoacrylate, E(A-A), exists both in an open and a closed conformation depending on the presence of monovalent cations, whereas the quinonoid, $E(Q_3)$, is predominantly in the closed state (13). Only the closed state of the β-subunit appears to be competent in the transmission of allosteric signals and, therefore, in stabilizing the closed, catalytically active conformation of the α-subunit. Through this mechanism, the catalytic activities of α- and β-subunits are finely tuned and kept in phase (4, 12, 13, 22).

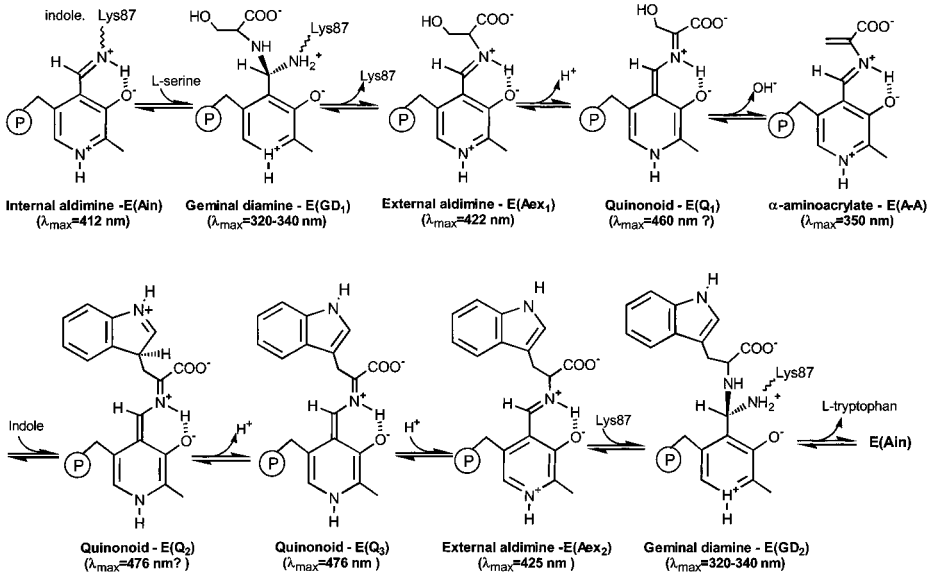
Determining the three-dimensional structure of an enzyme at different stages of the catalytic process is an important component of the effort to understand the structural basis of catalysis. In the case of tryptophan synthase, this is even more important since it would help to define the interplay between catalysis and regulation. Thus far, the structures of the internal aldimine (E(Ain), Scheme 1) and of the external aldimine ($E(Aex_1)$, Scheme 1) have been determined both in the absence and presence of α-subunit ligands (5, 23–26), providing structural information on the open and closed states of the α-subunit and on a partially open conformation of the β-subunit. Recently, the structure of the α-aminoacrylate in the presence of an α-subunit ligand has also been determined (26), unveiling the closed state of the β-subunit and shedding light on the pathway of communication between subunits. The last catalytic intermediate awaiting structural determination is the quinonoid $E(Q_3)$ (Scheme 1), formed in the reaction of the E(A-A) with indole. In solution, this species is formed transiently during the β-reaction (8, 27) or in low amounts upon reaction of the enzyme with the product L-tryptophan (28). In the crystal, this quinonoid does not accumulate appreciably (29). However, it is possible to form quinonoid species that are analogous to $E(Q_3)$ by reacting the aminoacrylate intermediate with nucleophiles such as β-mercaptoethanol (β-MSH)[1] (30),

* This work was supported by Italian National Research Council Grants 97.04377.CT14 and 98.001117.14/115.19978 (to A. M.) and by the Target Project on Biotechnology (to G. L. R.), the Italian Ministry of University and Scientific and Technological Research, and National Institutes of Health Grant GM55749 and National Science Foundation Grant DMB-87-03697 (to M. F. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This paper is dedicated to the memory of Professor Paolo Fasella.

¶ To whom correspondence should be addressed: Institute of Biochemical Sciences, Viale delle Scienze, University of Parma, 43100 Parma, Italy. Tel.: 39-0521-905138; Fax: 39-0521-905151; E-mail: biochim@ipruniv.cce.unipr.it.

[1] The abbreviations used are: β-MSH, β-mercaptoethanol; $E(Aex_1)$, enzyme-bound Schiff base of L-serine; E(A-A), enzyme-bound Schiff base of α-aminoacrylate; $E(Q_2)$ and $E(Q_3)$, enzyme-bound quinonoid



SCHEME 1. **Reaction intermediates formed in the β-active site of tryptophan synthase upon reaction with the substrates L-Ser and indole.**

indoline, phenylhydrazine, aniline, and small organic amines (31, 32).

Here, we have mainly investigated the accumulation and the stability of the quinonoid species formed by using the indole analog indoline in solution and crystal. In particular, since the equilibrium between the external aldimine and α-aminoacrylate is profoundly affected by pH, monovalent cations and α-subunit ligands (33, 34), we have studied how these factors influence the equilibrium between the α-aminoacrylate and the quinonoid species.

EXPERIMENTAL PROCEDURES

Materials—The tryptophan synthase $\alpha_2\beta_2$ complex from *Salmonella typhimurium* was expressed in an *Escherichia coli* strain containing the pEBA-10 plasmid encoding for the *S. typhimurium* genes and purified as described previously (35). Crystals of the enzyme were grown from PEG solutions as described previously (29, 36).

All chemicals were of the best available commercial quality and were used without further purification. The concentration of indoline was estimated on the basis of an extinction coefficient of 2,600 M^{-1} cm^{-1} at 289 nm (32).

Spectrophotometric Measurements—A solution containing the tryptophan synthase $\alpha_2\beta_2$ complex, 50–100 mM L-Ser, 25 mM bis-tris propane-HCl, at 20 °C, was titrated with increasing concentrations of nucleophilic reagents. Titrations were monitored using a Cary 219 spectrophotometer (Varian), interfaced to a personal computer for data storage. Cuvette holders were thermostated at 20 °C. Data were analyzed using a nonlinear least-squares fitting procedure available in the SigmaPlot software (Jandel).

Microspectrophotometry—Single crystals were mounted in a flow cell, placed on the thermostated stage of a Zeiss MPM03 microspectrophotometer, equipped with a ×10 Zeiss UV-visible ultrafluar objective. Polarized absorption spectra were collected with the electric vector of the linearly polarized light parallel to the extinction directions on the (210) flat face of monoclinic crystals (29). Experiments were carried out as described (29). Crystals of tryptophan synthase were suspended in either 25 mM bis-tris propane-HCl or 50 mM Bicine-NaOH containing 20% (w/v) PEG M_r 8000, 1 mM EDTA. The formation of quinonoids was monitored by recording spectra of crystals suspended in a solution containing 50 mM L-Ser and increasing concentrations of nucleophile, at 20 °C. The quinonoids are photosensitive chemical species that undergo accelerated decay when illuminated by strong light sources. To investigate quinonoid stability in the crystal, spectra were collected at intervals of 10–30 min, protecting the crystal from light between measurements.

RESULTS

Reaction of Indoline with the α-Aminoacrylate Intermediate in Solution—The reaction of indoline with *E*(A-A) leads to the formation of a metastable quinonoid species absorbing at 466 nm (32). This reaction was previously characterized under a unique set of experimental conditions (0.1 M potassium phosphate, pH 7.8, 22 °C) (32). As it was later shown that pH and monovalent cations strongly affect the equilibrium distribution of catalytic intermediates (14, 33, 34), we have investigated in detail the reaction of indoline as a function of pH in the absence and presence of either Na^+ or Cs^+. These ions were selected because their binding mode and their structural effects have been characterized by x-ray crystallography (37) and their influence on the equilibrium distribution between the external aldimine and the α-aminoacrylate are known (33).

A representative titration of the enzyme-serine system with increasing concentrations of indoline is shown in Fig. 1. From this and other similar titrations we obtained two parameters used for subsequent analysis: K_d, *i.e.* the apparent indoline dissociation constant under a given set of conditions,[2] and A_∞, the amplitude of the quinonoid band at saturating indoline concentration.

The apparent indoline dissociation constant is affected by the presence and nature of monovalent cations (Fig. 2*a*). At alkaline pH, the K_d measured in the presence of Cs^+ is slightly lower than in the absence of monovalent cations, whereas K_d measured in the presence of Na^+ is 6–10-fold lower (Fig. 2*a*; at pH values <7, K_d in the absence of monovalent cations becomes too high to be reliably measured). Furthermore, the apparent dissociation constant is significantly pH-dependent, both in the absence and presence of metal ions (Fig. 2*a*).

Monovalent cations also significantly increase A_∞ (Fig. 2*b*). Assuming that the extinction coefficient of $E(Q)_{Indoline}$ is cation-independent, this finding suggests that monovalent cations affect the equilibrium between $E(Q)_{Indoline}$ and *E*(A-A), in the presence of saturating indoline. This observation and the effects of Na^+ and Cs^+ on the indoline K_d explain the earlier observation that very little indoline quinonoid is formed in the

species formed upon reaction of *E*(A-A) with indole; $E(Q)_{Indoline}$ and $E(Q^H)_{Indoline}$, quinonoid species formed upon reaction of E(A-A) with indoline; PEG, polyethylene glycol; bis-tris, 2-[bis(2-hydroxyethyl)-amino]-2-(hydoxymethyl)-propane-1,3-diol; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; GP, D,L-α-glycerol-3-phosphate; λ_{max}, wavelength of maximum absorbance.

[2] K_d reflects the overall affinity of indoline for the β-active site, including all steps between noncovalent binding and formation of the quinonoid species.

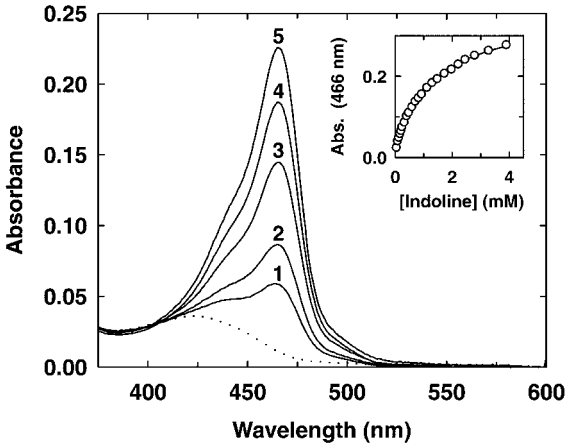


FIG. 1. **Titration of the serine-enzyme system with indoline.** Increasing concentrations of indoline were added to a solution containing 3.45 μM tryptophan synthase $\alpha_2\beta_2$ complex, 25 mM bis-tris propane, 50 mM L-Ser, 250 mM NaCl, pH 9.3, 20 °C. Spectra were recorded before (···) and after the addition of indoline at concentrations of 0.04 (*1*), 0.31 (*2*), 0.81 (*3*), 1.29 (*4*), and 2.2 mM (*5*). *Inset,* the absorbance change at 466 nm was fit to a binding isotherm with K_d of 1.4 ± 0.1 mM and an extrapolated maximum absorbance, A_∞ = 0.34 ± 0.05.

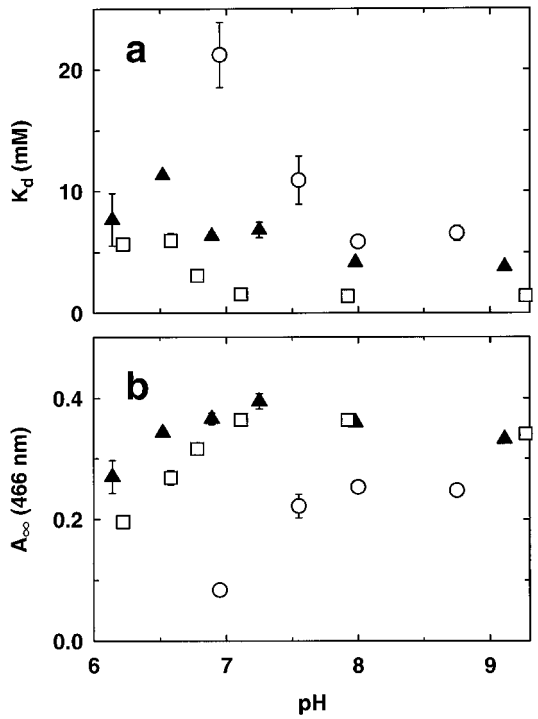


FIG. 2. **Effects of pH and monovalent cations on the formation of $E(Q)_{Indoline}$.** Titrations with indoline were carried out at different pH values in the absence of monovalent cations (○) and in the presence of either 250 mM NaCl (□) or 100 mM CsCl (▲). Other conditions were as described in the legend to Fig. 1. *Panel a,* pH dependence of K_d for indoline. *Panel b,* dependence of A_∞, the absorbance at 466 nm extrapolated at saturating indoline.

absence of monovalent cations (14). The increase in quinonoid formation with pH (Fig. 2*b*) parallels the increase in the apparent affinity for indoline (Fig. 2*a*). The pH dependence observed in the absence and presence of cations are markedly different (Fig. 2*b*), although, given the limited data set, a quantitative analysis of the pH profiles was not attempted.

Above pH 7, A_∞ values measured in the presence of either Na^+ or Cs^+ are identical within error and nearly pH independent. This suggests that, under these conditions, almost all the enzyme active sites contain the $E(Q)_{Indoline}$ intermediate.

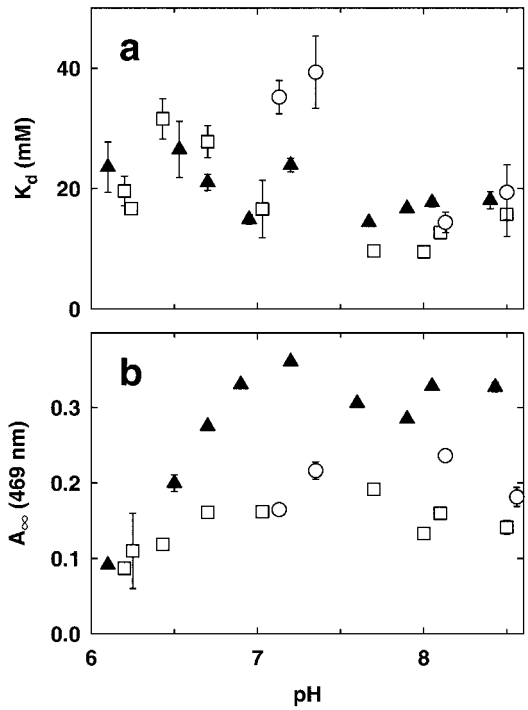


FIG. 3. **Effects of pH and monovalent ions on the reaction of β-mercaptoethanol with the enzyme-serine system.** Formation of $E(Q)_{\beta\text{-MSH}}$ was investigated as a function of pH in the absence (○) and presence of either 250 mM sodium chloride (□) or 100 mM cesium chloride (▲). The buffer was 50 mM bis-tris propane and the L-ser concentration was 50 mM. *Panel a* shows the dependence of the apparent dissociation constant; *panel b* shows the dependence of the extrapolated absorbance value at 469 nm.

Based on this assumption and on the known concentration of active sites in our assays, we estimate an extinction coefficient for the indoline quinonoid of ~53,000 M^{-1} cm^{-1}. This value is close to the extinction coefficients previously estimated for similar quinonoids (32, 38, 39).

Reaction of β-Mercaptoethanol with the α-Aminoacrylate Intermediate—To determine if the effects of ions and pH on the formation and accumulation of quinonoid species may depend on the structure of indoline, we investigated the reaction of the serine-enzyme system with a very different nucleophile, β-MSH. Attack of β-MSH on E(A-A) leads to the formation of an $E(Q)_{\beta\text{-MSH}}$ intermediate absorbing at 468 nm (2, 28, 30); this species is less stable than the indoline quinonoid and decays with formation of *S*-hydroxyethyl-L-cysteine (40). In our experiments, the concentration of nucleophile was kept below 50 mM to avoid the effects of β-MSH acting as a cosolvent (41).

The results of β-MSH titrations, carried out as a function of pH in the absence and presence of monovalent cations, are summarized in Fig. 3. K_d showed little dependence on pH. Furthermore, virtually identical K_d values were observed at high pH both in the absence of cations and in the presence of either Na^+ or Cs^+ (Fig. 3*a*). This observation suggests that the effect of monovalent cations on indoline binding (Fig. 2*a*) depends on the structure of this aromatic nucleophile. In particular, whereas indoline presumably binds noncovalently to a hydrophobic pocket in the β-active site prior to reacting with the α-aminoacrylate, it is possible that formation of the β-MSH quinonoid may proceed without formation of a stable noncovalent adduct.

Cesium ions were more effective than sodium ions at favoring accumulation of the β-MSH quinonoid (Fig. 3*b*). This finding is consistent with previous observations showing that different metal ions stabilize preferentially different reaction

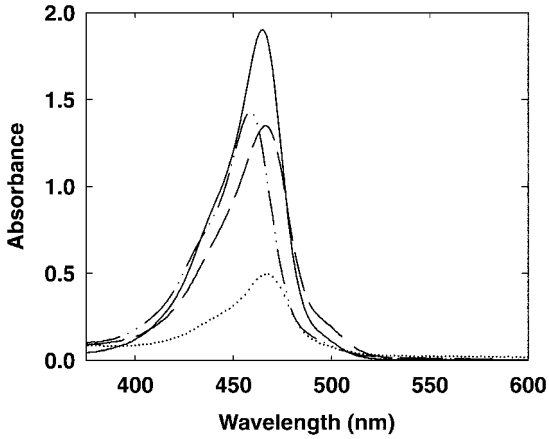


FIG. 4. **Polarized absorbance spectra of quinonoid species in the crystal.** Crystals of tryptophan synthase were suspended in a medium containing 50 mM L-Ser, 20% (w/v) PEG 8000, 1 mM EDTA, 50 mM Bicine-Na buffer, pH 7.8, and one of the following nucleophiles: 10 mM indoline (——), 10 mM phenylhydrazine (– – –); 10 mM aniline (···), 50 mM *N*-methylhydroxylamine (– –). Spectra were recorded with the electric vector of the linearly polarized light parallel to an extinction direction of the crystal.

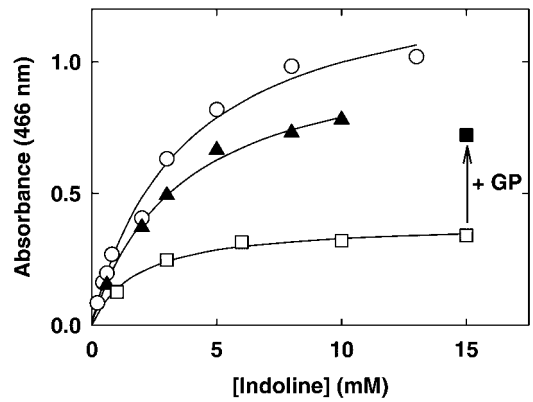


FIG. 5. **Reaction of indoline with tryptophan synthase crystals in the absence and presence of monovalent cations.** Crystals of tryptophan synthase were suspended in a solution containing increasing indoline concentrations, 50 mM L-ser, 20% (w/v) PEG 8000, 25 mM bis-tris propane buffer, pH 8, in the absence of monovalent metal ions (○) and in the presence of 100 mM CsCl (▲), 250 mM NaCl (□), 250 mM NaCl plus 50 mM glycerol-3-phosphate (■). Polarized absorption spectra were recorded within 15–30 min after addition of indoline. Due to the relative instability of $E(Q)_{Indoline}$, a separate crystal was used for each indoline concentration. Data from different crystals were normalized on the basis of the spectra of the parent internal aldimine species. The *curves* drawn through the data points are best fits to the equation for a binding hyperbolic isotherm, yielding the K_d values listed in Table I.

intermediates (14, 33). In the presence of Na^+ or Cs^+, A_∞ increased with pH up to about pH 7 and remained nearly stable thereafter (Fig. 3*b*), similarly to what observed with the indoline quinonoid (Fig. 2*b*).

Formation of Quinonoid Intermediates in the Crystal—In a previous investigation on the reactivity of tryptophan synthase in the crystalline state, we reported formation of the β-MSH quinonoid in the presence of α-subunit ligands such as indole-3-propanol phosphate (Ref. 29) (a modest amount of quinonoid could also be accumulated by reacting the enzyme with the product analog dihydro-5-fluoro-L-tryptophan). Here, we have characterized the formation of quinonoid intermediates upon reaction of E(A-A) with a series of other nucleophiles: indoline, phenylhydrazine, *N*-methylhydroxylamine, aniline (Fig. 4) or *O*-methylhydroxylamine (not shown). These quinonoids exhibit very high extinction coefficients and roughly similar band shapes, but differ significantly in λ_{max} (11, 14, 32). The polarized absorption spectra of these intermediates in the crystal (Fig. 4) closely resembled the spectra obtained in solution under similar experimental conditions.

Accumulation and Stability of the Indoline Quinonoid in the Crystal—Titrations with indoline were carried out at pH 8.0 in the absence and presence of either cesium or sodium ions (Fig. 5). The observed indoline K_d values were comparable to those measured with the soluble enzyme under similar conditions, and were scarcely affected by the presence of monovalent cations (Table I). However, the maximal amount of $E(Q)_{Indoline}$ significantly depended on the presence or absence of monovalent cations (Fig. 5). In sharp contrast with the behavior observed in solution, the presence of Na^+ markedly diminished the accumulation of quinonoid. When the α-subunit ligand D,L-α-glycerol-3-phosphate (GP) was present concomitantly with Na^+, the amount of $E(Q)_{Indoline}$ increased and was as high as in the presence of Cs^+ (Fig. 5).

The effect of pH on the accumulation of the quinonoid species was examined by recording polarized absorption spectra in the presence of 3 mM indoline at different pH values (6 to 9), either in the absence of cations or in the presence of Na^+ or Cs^+. In all cases, accumulation of the quinonoid increased with pH until it reached a maximum at about pH 8 and remained stable up to pH 9 (data not shown). Such observed pH dependence, measured in the presence of a single concentration of indoline, may arise from the same effects observed in solution: the effect of pH on the indoline K_d and the pH dependence of the maximum amount of $E(Q)_{Indoline}$ that can be accumulated.

TABLE I

Apparent affinity of indoline and rates of quinonoid decay in the crystal in the presence of different effectors

Indoline K_d values for the crystalline enzyme at pH 8 were obtained from the data shown in Fig. 5. For half-life measurements, the $E(Q)_{Indoline}$ intermediate was formed by reacting tryptophan synthase crystals with 50 mM L-Ser and 15 mM indoline, at pH 7.8, 20 °C, and its decay was followed by collecting spectra at intervals of 10–30 min.

Effector	Indoline K_d		Quinonoid half-life in the crystal
	Crystal	Solution	
	mM		*min*
None	3.7 ± 0.9	5.8 ± 0.5	150
100 mM CsCl	3.6 ± 1.0	4.2 ± 0.2	180
250 mM NaCl	1.8 ± 1.2	1.4 ± 0.1	300
250 mM NaCl + 50 mM GP	ND[a]	ND	>600

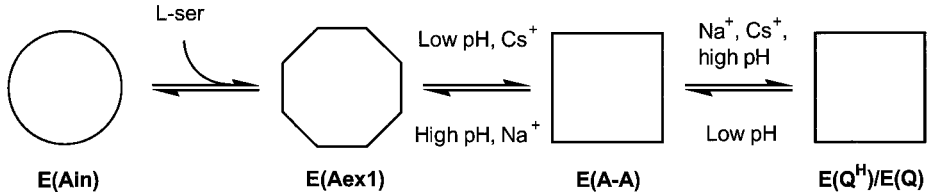
[a] ND, not determined.

To carry out an x-ray crystallographic analysis of the $E(Q)_{Indoline}$ species, it is essential not only to accumulate this intermediate to the highest extent, but also to make sure that the quinonoid is reasonably stable as a function of time. Decay of $E(Q)_{Indoline}$ in the crystal was monitored at room temperature in the absence and presence of monovalent cations and/or of the α-subunit ligand GP (Table I). The observed half-lives were on the order of a few hours, confirming preliminary observations (42). Decay of $E(Q)_{Indoline}$ was particularly slow in the presence of Na^+ and GP (Table I). The quinonoid species formed by phenylhydrazine and aniline also decayed on time scales of hours (not shown).

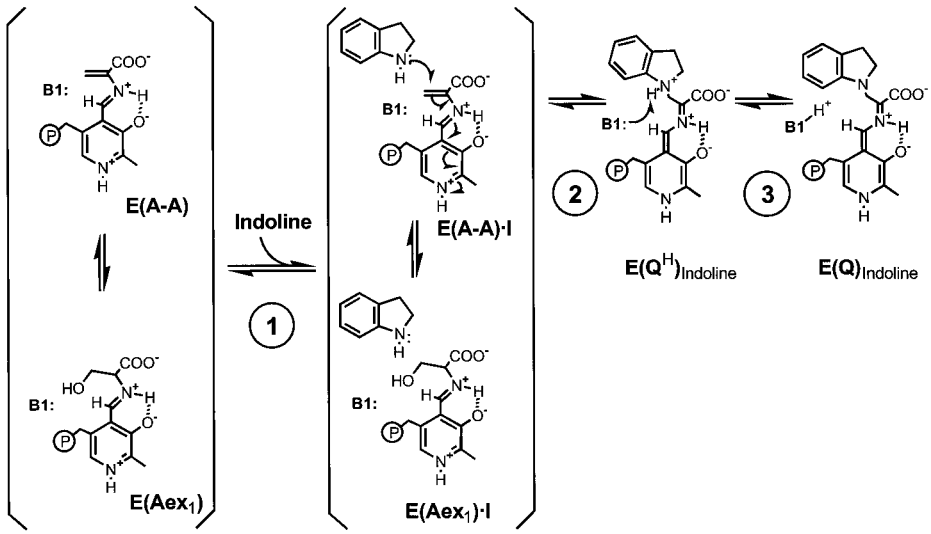
DISCUSSION

The determination of the three-dimensional structures of chemical and conformational intermediates in enzyme-catalyzed reactions is essential for understanding the relationships between structure and biological function. Solution investigations of the αβ reaction of tryptophan synthase have revealed an essential interplay between chemical and conformational events (1, 3, 4). Catalytic steps and conformational changes are linked by an intricate set of allosteric interactions, which allow

SCHEME 2. **Conformation equilibria of the tryptophan synthase β-subunit.** The open state is schematically represented by a *circle*, the partially open conformation is represented by a *hexagon*, and the closed conformation is represented by a *square*.



SCHEME 3. **Possible mechanism of formation of the indoline quinonoid in the tryptophan synthase β-active site.** *B1* represents a ionizable group involved in deprotonation of the initial product of the nucleophilic attack.



achievement of efficient catalysis at the α- and β-sites as well as regulation of indole channeling (4, 43). The present structural data base pertaining to the αβ reaction (5, 23–26, 37) has introduced critically important information about the architecture of the protein. However, both the nature of the chemical bonding interactions that result in catalysis and the allosteric regulation of channeling and catalysis remain unclear. Among the several facets of the catalytic and regulatory cycle that are still poorly understood are the following: (*a*) the structural basis of catalysis at the α- and β-sites; (*b*) the conformational events that trigger activation of the α-site; (*c*) the conformational changes induced by monovalent cations that establish allosteric communication between the α- and β-sites, and activate the β-site; and (*d*) the structure of a tryptophan synthase quinonoidal intermediate, a species that also stabilizes the activated conformation of the α-site.

The partially closed structures determined for the $E(Aex_1)$ and $E(Aex_2)$ complexes of the βK87T mutant appear not to perturb the α-site (23), a conclusion in agreement with the finding that the wild-type complexes of these species do not activate the α-site (12), nor do they alter the dynamic properties of the α-site (9). The x-ray structure of *E*(A-A) complexed with 5-fluoroindole propanol phosphate (26) reveals a completely closed conformation that is different from the βK87T complexes. Whereas structures of the Na^+, K^+, and Cs^+ forms of the enzyme and several enzyme intermediates (or analogues thereof) have been solved, the structures of cation-free forms of the enzyme have not been reported, and the origins of the monovalent cation effects remain structurally obscure.

The Tryptophan Synthase Quinonoids—The β-reaction catalyzed by tryptophan synthase involves at least nine covalent transformations and eight covalent intermediates. The bond scission and formation steps involve C-N single and double bonds, a C-O single bond, C-H bonds, N-H bonds, O-H bonds, and a C=C double bond. Scission of the C-H bond at the C-α of $E(Aex_1)$ is energetically difficult and rate determining for the β- and αβ-reactions under steady-state conditions (16). The proton removed from C-α of $E(Aex_1)$ has been postulated to be trapped within a low barrier hydrogen bond at the β-site in the *E*(A-A) state (44). Formation of *E*(A-A) is the chemical trigger that switches the protein to the closed state and brings about activation of the α-site (12). The reversal of this conformational switch occurs when $E(Q_3)$ is converted to $E(Aex_2)$ (13). Consequently, knowledge of the relationship between structure and function in the tryptophan synthase system will be significantly advanced by further work on the structures of the α-aminoacrylate and quinonoid complexes, both with and without monovalent cations bound to the β-site, and with substrate or effectors bound to the α-site. Toward this end, we have established conditions under which the indoline quinonoid is stabilized in the crystal.

The indole analogues, aniline, phenylhydrazine, and methylated hydroxylamines were all shown to be potential candidates for the determination of a quinonoid structure. However, indoline appears to be the system of choice for several reasons: (*a*) indoline is a close structural homologue of indole; (*b*) indoline is an alternative substrate for tryptophan synthase, yielding the artificial amino acid, dihydroiso-L-tryptophan (32); (*c*) indoline is a well established kinetic and mechanistic probe of the *E*(A-A) species (6, 12, 14, 16, 21, 32); and (*d*) as discussed below, a suitable combination of monovalent cations, α-site ligand and pH conveys remarkable stability to the indoline quinonoid at room temperature, both in solution and in the crystalline state. Nevertheless, different conditions are required for optimizing quinonoid yield and stability in solution and crystal.

Influence of pH and Monovalent Cations on the Chemical and Conformation States of Tryptophan Synthase—The role of pH and monovalent cations in modulating the chemical and conformational equilibria involved in the formation of the indoline quinonoid is summarized in Scheme 2. The equilibrium distribution between $E(Aex_1)$ and *E*(A-A) is controlled by two ionizations with pK_a values of 7.8 and 10.2 (34). Low pH favors the accumulation of *E*(A-A), high pH favors accumulation of $E(Aex_1)$ and α-subunit ligands strongly favor *E*(A-A) at all pH

values. Different protein conformations are associated with $E(Aex_1)$ and E(A-A), the former residing in a partially open state and the latter in the closed state. Both $E(Q^H)_{Indoline}$/$E(Q)_{Indoline}$ and E(A-A) are predominantly in a closed conformation (13, 14, 22).

The reaction of indoline with the tryptophan synthase-serine system involves at least three steps (Scheme 3); formation of a Michaelis complex at the β-subunit (step 1, Scheme 3), nucleophilic attack on the β-carbon of E(A-A), to give a quinonoid intermediate, $E(Q^H)_{Indoline}$ (step 2, Scheme 3; 32), which may be subsequently deprotonated by a basic group (B1, Scheme 3) to give $E(Q)_{Indoline}$. These steps are accompanied by adjustment of the α- and β-subunit conformational equilibria to binding and reaction. In contrast to the reaction of E(A-A) with indole, formation of the indoline quinonoid C-N bond is freely reversible (6). Since further reaction is very slow (32), the apparent affinity for indoline and the accumulation of quinonoid depend on the equilibria described in Scheme 3 and on the factors that influence these equilibria (*e.g.* pH, monovalent cations, and α-site ligands).

pH Effects on the Quinonoid Yield and the Apparent Affinity of E(A-A) for Indoline in Solution—At equilibrium, in Stage I of the β-reaction, E(A-A) predominates at low pH, whereas the external aldimine is stabilized by high pH values (34). If this effect were fully maintained in the presence of saturating indoline, it would antagonize the formation of quinonoid at high pH. Nevertheless, the accumulation of the indoline quinonoid increases with pH up to about 7.5 and remains nearly constant thereafter (Fig. 2*b*). This finding suggests that the equilibrium between $E(Aex_1)$ and E(A-A) shifts toward the latter when indoline binds. The binding of indole (8) or indole analogs (11) is known to induce a redistribution of intermediates bound to the β-active site.

Since the pH dependence of quinonoid accumulation is very similar for indoline (pK_a ~5; Ref. 13) and for β-MSH (pK_a ~9.5; Ref. 41), it is unlikely that quinonoid formation is controlled by the ionization properties of the nucleophile.[3] This observation suggests that the pH dependence reflects the ionization of a protein residue. Protonation of the basic residue (B1 Scheme 3) that abstracts a proton from $E(Q^H)_{Indoline}$ (45) would decrease quinonoid accumulation. A similar proton transfer is known to occur for the indole system.

The same factors that increase quinonoid stability also increase the apparent affinity for indoline. Hence, the modest effect of pH on K_d may result from a trade off: the pH dependence of the $E(Aex_1) \leftrightarrow E$(A-A) equilibrium should disfavor the noncovalent binding step at high pH, but this effect might be more than countered by the stabilization of the quinonoid species at high pH.

Effects of Monovalent Cations on the Quinonoid Yield and the Apparent Affinity of E(A-A) for Indoline in Solution—Sodium and cesium ions bind to the same site in the β-subunit, about 8 Å from pyridoxal 5′-phosphate (37). A rotation of about 1° of the β-subunit with respect to the α-subunit was observed when either Cs^+ or K^+ is bound in place of Na^+. Phosphorescence and NMR line width measurements suggest that both ions cause the β-active site to become less flexible (33, 46). Yet the functional effects of these cations are distinct: in the absence of added nucleophiles, Cs^+ perturbs the $E(Aex_1) - E$(A-A) equilibrium, strongly favoring E(A-A), and likely stabilizes a closed conformation of the β-subunit, whereas Na^+ favors $E(Aex_1)$ and the "partially closed" conformation. In the absence of monovalent cations, the predominant species is E(A-A) (33).

In the presence of indoline, Cs^+ favors a higher accumulation of quinonoid at all pH values tested (Figs. 2*b* and 3*b*) and Na^+ ions favor accumulation of the indoline quinonoid to nearly the same extent. Furthermore, both monovalent cations tested decrease the apparent K_d for indoline. This finding suggests that Na^+ and Cs^+ within a closed conformation act similarly despite the different effects these ions have on the equilibria between open and closed states. It is also possible that metal ions modulate the pK_a of the residue responsible for quinonoid deprotonation (step 3, Scheme 3). This would be consistent with the different pH dependence of quinonoid yield (A_∞) in the presence and absence of monovalent cations (Fig. 2*b*).

Stabilization of the Indoline Quinonoid in the Crystalline State—The results presented in Fig. 5 and in Table I establish both the conditions under which the crystalline indoline quinonoid may be formed and the extent to which stabilization may be achieved through the action of monovalent cation binding, the effects of pH, and the binding of GP. In contrast to the behavior observed in solution, in the absence of other effectors, Na^+ is ineffective in stabilizing $E(Q)_{Indoline}$; indeed, the highest yields of the quinonoid were obtained in the cation-free system (Fig. 5). However, both the combination of Na^+ and GP, or Cs^+ alone, were found to be relatively effective. Analysis of the light absorbing properties of the quinonoid crystals measured along the direction of maximum absorbance is consistent with a high occupancy of β-sites by the indoline quinonoid.

The apparent half-lives for $E(Q)_{Indoline}$ decay, shown in Table I, establish that the combination of Na^+ and GP gives the greatest stabilization (by a factor >4-fold over the stability determined in the absence of effectors). Together, these observations concerning the influence of effectors and pH on $E(Q)_{Indoline}$ yield and stability indicate that the combination of Na^+ and GP at pH 8 to 9 provides a highly stable quinonoid species that should be an excellent candidate for structure determination via single crystal x-ray diffraction.

These results emphasize the utility of conducting spectroscopic analyses of protein-ligand interactions in the crystalline state to direct x-ray diffraction studies (47). This approach is particularly critical when dealing with catalytic intermediates such as quinonoids species that are metastable and have so far eluded structural determination.

Acknowledgment—We thank Dr. Edith W. Miles (National Institutes of Health) for the gift of the *E. coli* strain containing the plasmid encoding *S. typhimurium* tryptophan synthase genes.

REFERENCES

1. Yanofsky, C., and Crawford, I. P. (1972) in *The Enzymes* (Boyer, P. D., ed) Vol. VII, pp. 1–31, Academic Press, New York
2. Miles, E. W. (1979) *Adv. Enzymol.* **49,** 127–186
3. Miles, E. W. (1991) *Adv. Enzymol.* **64,** 93–172
4. Pan, P., Woehl, E., and Dunn, M. F. (1997) *Trends Biochem. Sci.* **22,** 22–27
5. Hyde, C. C., Ahmed, S. A., Padlan, E. A., Miles, E. W., and Davies, D. R. (1988) *J. Biol. Chem.* **263,** 17857–17871
6. Dunn, M. F., Aguilar, V., Brzovic, P. S., Drewe, W. F., Houben, K. F., Leja, C. A., and Roy, M. (1990) *Biochemistry* **29,** 8598–8607
7. Drewe, W. F., and Dunn, M. F. (1985) *Biochemistry* **24,** 3977–3987
8. Drewe, W. F., and Dunn, M. F. (1986) *Biochemistry* **25,** 2495–2501
9. Strambini, G. B., Cioni, P., Peracchi, A., and Mozzarelli, A. (1992) *Biochemistry* **31,** 7535–7542
10. Vaccari, S., Benci, S., Peracchi, A., and Mozzarelli, A. (1996) *Biophys. Chem.* **61,** 9–22
11. Houben, K., and Dunn, M. F. (1990) *Biochemistry* **29,** 2421–2429
12. Brzovic, P. S., Ngo, K., and Dunn, M. F. (1992) *Biochemistry* **31,** 3831–3839
13. Leja, C. A., Woehl, E. U., and Dunn, M. F. (1995) *Biochemistry* **34,** 6552–6561
14. Woehl, E. U., and Dunn, M. F. (1995) *Biochemistry* **34,** 9466–9476
15. Woehl, E., and Dunn, M. F. (1999) *Biochemistry* **38,** 7118–7130
16. Woehl, E., and Dunn, M. F. (1999) *Biochemistry* **38,** 7131–7141
17. Anderson, K. S., Miles, E. W., and Johnson, K. A. (1991) *J. Biol. Chem.* **266,** 8020–8033
18. Kirschner, K., Wiskocil, R., Foehn, M., and Rezeau, L. (1975) *Eur. J. Biochem.* **60,** 513–524
19. Kirschner, K., Weischet, W., and Wiskocil, R. (1975) in *Protein-Ligand Interactions* (Sund, H., and Blauer, G., eds) pp. 27–42, Walter de Gruyter and Co., Berlin

[3] If β-MSH binds to E(A-A) as the neutral thiol, with transfer of the thiol proton to B1 occurring prior to, or during, the nucleophilic attack on E(A-A), then, the pK_a of β-MSH would not influence quinonoid yield in the pH range studied (pH 6 to 9).

20. Kirschner, K., Lane, A. N., and Strasser, A. W. N. (1991) *Biochemistry* **30,** 472–478
21. Brzovic, P. S., Sawa, Y., Hyde, C. C., Miles, E. W., and Dunn, M. F. (1992) *J. Biol. Chem.* **267,** 13028–13038
22. Pan, P., and Dunn, M. F. (1996) *Biochemistry* **35,** 5002–5013
23. Rhee, S., Parris, K. D., Hyde, C. C., Ahmed, S. A., Miles, E. W., and Davies, D. R. (1997) *Biochemistry* **36,** 7664–7680
24. Rhee, S., Miles, E. W., Mozzarelli, A., and Davies, D. R. (1998) *Biochemistry* **37,** 10653–10659
25. Rhee, S., Miles, E. W., and Davies, D. R. (1998) *J. Biol. Chem.* **273,** 8553–8555
26. Schneider, T. R., Gerhardt, E., Lee, M., Liang, P.-H., Anderson, K. S., and Schlichting, I. (1998) *Biochemistry* **37,** 5394–5406
27. Lane, A. N., and Kirschner, K. (1983) *Eur. J. Biochem.* **129,** 571–582
28. Miles, E. W. (1980) in *Biochemical and Medical Aspects of Trytophan Metabolism* (Hayaishi, O., Ishimura, Y., and Kido, R., eds) Elsevier/North Holland, Amsterdam
29. Mozzarelli, A., Peracchi, A., Rossi, G. L., Ahmed, S. A., and Miles, E. W. (1989) *J. Biol. Chem.* **264,** 15774–15780
30. Goldberg, M. E., and Baldwin, R. L. (1967) *Biochemistry* **6,** 2113–2119
31. Dunn, M. F., Aguilar, V., Drewe, W. F., Jr., Houben, K., Robustell, B., and Roy, M. (1987) *Indian J. Biochem. Biophys.* **24,** (suppl.) 44–51
32. Roy, M., Keblawi, S., and Dunn, M. F. (1988) *Biochemistry* **27,** 6698–6704
33. Peracchi, A., Mozzarelli, A., and Rossi, G. L. (1995) *Biochemistry* **34,** 9459–9465
34. Peracchi, A., Bettati, S., Mozzarelli, A., Rossi, G. L., Miles, E. W., and Dunn, M. F. (1996) *Biochemistry* **35,** 1872–1880
35. Yang, L., Ahmed, S. A., and Miles, E. W. (1996) *Protein Exp. Purif.* **8,** 126–136
36. Ahmed, S. A., Miles, E. W., and Davies, D. R. (1985) *J. Biol. Chem.* **260,** 3716–3718
37. Rhee, S., Parris, K. D., Ahmed, S. A., Miles, E. W., and Davies, D. R. (1996) *Biochemistry* **35,** 4211–4221
38. Metzler, C., Harris, A. G., and Metzler, D. E. (1988) *Biochemistry* **27,** 4923–4933
39. Roy, M., Miles, E. W., Phillips, R. S., and Dunn, M. F. (1988) *Biochemistry* **27,** 8661–8669
40. Miles, E. W., Hatanaka, M., and Crawford, I. P. (1968) *Biochemistry* **7,** 2742–2753
41. Ahmed, S. A., McPhie, P., and Miles, E. W. (1996) *J. Biol. Chem.* **271,** 29100–29106
42. Rossi, G. L., Mozzarelli, A., Peracchi, A., and Rivetti, C. (1992) *Philos. Trans. R. Soc. Lond. A Math. Phys. Sci.* **340,** 191–207
43. Miles, E. W., Rhee, S., and Davies, D. R. (1999) *J. Biol. Chem.* **274,** 12193–12196
44. Hur, O., Leja, C., and Dunn, M. F. (1996) *Biochemistry* **35,** 7378–7386
45. Phillips, R. S., Miles, E. W., and Cohen, L. A. (1984) *Biochemistry* **23,** 6228–6234
46. Schnackerz, K., and Mozzarelli, A. (1998) *J. Biol. Chem.* **273,** 33247–33253
47. Mozzarelli, A., and Rossi, G. L. (1996) *Annu. Rev. Biophys. Biomol. Struct.* **25,** 343–365

Effect of pH and Monovalent Cations on the Formation of Quinonoid Intermediates of the Tryptophan Synthase $\alpha_2\beta_2$ Complex in Solution and in the Crystal

Andrea Mozzarelli, Alessio Peracchi, Barbara Rovegno, Giovanna Dalè, Gian Luigi Rossi and Michael F. Dunn

J. Biol. Chem. 2000, 275:6956-6962.
doi: 10.1074/jbc.275.10.6956