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-aminoacylate Schiff base with different nucleophiles, focusing mainly on the substrate analog indole and β-mercaptoethanol. In solution, both monovalent cations (Ca\(^{2+}\) 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 ionicizable 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 αβ\(_2\) 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 aldimes 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-aminoacylate, E(A-A), exists both in an open and a closed conformation depending on the presence of monovalent cations, whereas the quinonoid, E(Q3), 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 aldime (E(Ain), Scheme 1) and of the external aldime (E(Aex1), 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-aminoacylate in the presence of an α-subunit ligand has also been determined (28), 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(Q3) (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(Q3) by reacting the aminoacylate intermediate with nucleophiles such as β-mercaptoethanol (β-MSH) (30),

1 The abbreviations used are: β-MSH, β-mercaptoethanol; E(Aex1), enzyme-bound Schiff base of L-serine; E(A-A), enzyme-bound Schiff base of α-aminoacylate; E(Q2) and E(Q3), enzyme-bound quinonoid
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-aminoacrylalate 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 αβ complex from Salmonella typhimurium was expressed in an Escherichia coli strain containing the pERA-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 (36). Crystals of the enzyme were grown from PEG solutions as described previously (36). Crystals of the enzyme were grown from PEG solutions as described previously (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 αβ 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 ultrafluor 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 M₅₀₀₀, 1 mM EDTA. The formation of quinonoids was estimated on the basis of an extinction coefficient of 2,600 M⁻¹ cm⁻¹ at 289 nm (32).

**RESULTS**

**Reaction of Indoline with the α-Aminoacylate 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 mM 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 aldime 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, and $A_\infty$, 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. 2a). 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. 2a). 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 aldime and the α-aminoacrylate are known (33).

Assuming that the extinction coefficient of $E(Q)_{\text{Indoline}}$ is constant independent, this finding suggests that monovalent cations affect the equilibrium between $E(Q)_{\text{Indoline}}$ 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

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**Scheme 1.** Reaction intermediates formed in the β-active site of tryptophan synthase upon reaction with the substrates L-Ser and indole.
absence of monovalent cations (14). The increase in quinonoid formation with pH (Fig. 2b) parallels the increase in the apparent affinity for indoline (Fig. 2a). The pH dependence observed in the absence and presence of cations are markedly different (Fig. 2b), 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 \( E(Q) \) intermediate.

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 \( 53,000 \) \( \text{M}^{-1} \text{cm}^{-1} \). This value is close to the extinction coefficients previously estimated for similar quinonoids (32, 38, 39).

**Reaction of \( \beta \)-Mercaptoethanol with the \( \alpha \)-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, \( \beta \)-MSH. Attack of \( \beta \)-MSH on \( E(A-A) \) leads to the formation of an \( E(Q)_{\beta-MSH} \) 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 \( \beta \)-MSH acting as a cosolvent (41).

The results of \( \beta \)-MSH titrations, carried out as a function of pH in the absence and the presence of either Na\(^+\) or Cs\(^+\) (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. 3a). This observation suggests that the effect of monovalent cations on indoline binding (Fig. 2a) depends on the structure of this aromatic nucleophile. In particular, whereas indoline presumably binds noncovalently to a hydrophobic pocket in the \( \beta \)-active site prior to reacting with the \( \alpha \)-aminoaCRYlate, it is possible that formation of the \( \beta \)-MSH quinonoid may proceed without formation of a stable noncovalent adduct.

Cesium ions were more effective than sodium ions at favoring accumulation of the \( \beta \)-MSH quinonoid (Fig. 3b). This finding is consistent with previous observations showing that different metal ions stabilize preferentially different reaction
intermediates (14, 33). In the presence of Na⁺ or Cs⁺, $A_\infty$ increased with pH up to about pH 7 and remained nearly stable thereafter (Fig. 3b), similarly to what observed with the indoline quinonoid (Fig. 2b).

**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 $E_i(A-A)$ 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 $\lambda_{\text{max}}$ (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**—Titration 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 metal ions (Table I). However, the maximal amount of $E_i(Q)$ 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⁺ markedely 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 $E_i(Q)$ 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 $E_i(Q)$ indoline that can be accumulated.

To carry out an x-ray crystallographic analysis of the $E_i(Q)$ 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 $E_i(Q)$ 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 $E_i(Q)$ 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
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 quinonoid intermediate, a species that also stabilizes the activated conformation of the α-site.

The partially closed structures determined for the E(Aexx) and E(Aexx) 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-fluorindole 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(Aexx) 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(Aexx) 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(Q2) is converted to E(Aexx) (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-aminoacyl 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, indole appears to be the system of choice for several reasons: (a) indole is a close structural homologue of indole; (b) indole is an alternative substrate for tryptophan synthase, yielding the artificial amino acid, dihydroiso-1-trypthanyl (32); (c) indole 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 Conformational 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(Aexx) and E(A-A) is controlled by two ionizations with pKₐ values of 7.8 and 10.2 (34). Low pH favors the accumulation of E(A-A), high pH favors accumulation of E(Aexx) 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 $E(QH)_{\text{Indoline}}$ 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 $\beta$-subunit (step 1, Scheme 3), nucleophilic attack on the $\beta$-carbon of $E(A-A)$, to give a quinonoid intermediate, $E(QH)_{\text{Indoline}}$ (step 2, Scheme 3; 32), which may be subsequently deprotonated by a basic group (B1, Scheme 3) to give $E(Q)_{\text{Indoline}}$. These steps are accompanied by adjustment of the $\alpha$- and $\beta$-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 $\alpha$-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 $\beta$-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. 2b). 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 $\beta$-active site.

Since the pH dependence of quinonoid accumulation is very similar for indoline (pK$_B$ = 5; Ref. 13) and for $\beta$-MSH (pK$_B$ = 9.5; Ref. 41), it is unlikely that quinonoid formation is controlled by the ionization properties of the nucleophile. 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 $E(QH)_{\text{Indoline}}$ (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 $E(Aex_1) \rightleftharpoons E(A-A)$ 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 $\beta$-subunit, about 8 Å from pyridoxal 5'-phosphate (37). A rotation of about 1° of the $\beta$-subunit with respect to the $\alpha$-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 $\beta$-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 $E(Aex_1) \rightleftharpoons E(A-A)$ equilibrium, strongly favoring $E(A-A)$, and likely stabilizes a closed conformation of the $\beta$-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. 2b and 3b) 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$_B$ of the residue responsible for quinonoid deprotonation (step 3, Scheme 3). This would be consistent with the different pH dependence of quinonoid yield ($A_\lambda$) in the presence and absence of monovalent cations (Fig. 2b).

**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 $E(Q)_{\text{Indoline}}$; 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 $\beta$-sites by the indoline quinonoid.

The apparent half-lives for $E(Q)_{\text{Indoline}}$ 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 $E(Q)_{\text{Indoline}}$ 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 quinonoid species that are metastable and have so far eluded structural determination.

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3 If $\beta$-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$_B$ of $\beta$-MSH would not influence quinonoid yield in the pH range studied (pH 6 to 9).
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