One-Electron Reduction of Flavoproteins: Pulse Radiolysis of Chicken Egg White Riboflavin Binding Protein†

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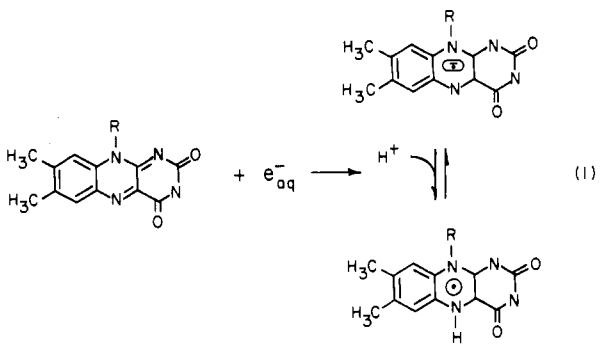
ABSTRACT: Reduction of the chicken egg white riboflavin binding protein by the hydrated electron results in competitive formation of both a disulfide–electron adduct and an anionic flavin semiquinone bound to the protein. The former decays to products that cannot be observed under the conditions of our experiments. The latter is rapidly protonated to the stable neutral semiquinone. The pH dependence of the rate constant associated with this protonation suggests that an acid/base group on the protein donates a proton to the anionic semiquinone.

Rapid protein reactions linked to an initiating electron transfer can be studied by pulse radiolysis, in which primary free radicals such as the hydrated electron (e_{aq})[1] are produced in a pulse of 1 μs or less. The methodology and some of the reactions observed with pulse radiolysis have been reviewed elsewhere (Matheson & Dorfman, 1969; Adams & Wardman, 1977; Klapper & Faraggi, 1979).

We are currently interested in the one-electron reduction of flavoproteins to form the protein-bound flavin semiquinone. In aqueous solution, the riboflavin semiquinone has a pK_a near 8.3 (Land & Swallow, 1969). Since the oxidized flavin is a stronger acid than the semiquinone, one-electron reduction at neutral pHs must be accompanied by protonation (eq 1).



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Questions of how and from where the proton reaches the semiquinone arise when the prosthetic group is buried within a protein, as in the case of flavodoxin (Mayhew & Ludwig, 1975).

In a previous pulse radiolysis study with flavodoxin, we have found that formation of the stable, protonated (hence, zwitterionic) semiquinone on the protein appears to involve a minimum of four processes (Faraggi & Klapper, 1979). Because of this complexity, we could not establish the semiquinone anion as a reaction intermediate. In the hope of observing the conversion from anionic to neutral semiquinone, we investigated the reduction of the riboflavin binding protein (RBP) from chicken egg white. RBP is plentiful in the whites of eggs, functioning perhaps as a riboflavin storage/transport protein. It can be obtained in relatively large quantities with a minimum of effort, and the flavin semiquinone is stabilized in its neutral form (Massey et al., 1978). We present here our results on the e_{aq}, single-electron reduction of RBP, which may serve as an analogy for the same reaction in flavin-containing redox proteins such as flavodoxin. We have found that the anionic flavin semiquinone is an immediate precursor of the neutral species.

Materials and Methods

The methods of protein pulse radiolysis have been discussed elsewhere (Klapper & Faraggi, 1979). Riboflavin binding protein was obtained from chicken egg white by a modification of Becvar's (1973) method. The whites of 10-dozen fresh eggs were diluted with an equal volume of 0.1 M sodium acetate buffer, pH 5.3. After addition of 50 mg of riboflavin and a stirring for 10–15 min, the suspension was strained through cheesecloth. DEAE-cellulose was added in small batches as a thick slurry. After each addition, the absorbance at 455 nm was measured, and addition of the DEAE-cellulose was stopped when that absorbance decreased by minimal amounts. After centrifugation, the DEAE-cellulose was resuspended in a minimal volume of the same buffer and packed into a glass column. The protein was eluted with 0.1 M CH_3COONa/0.2 M NaCl, pH 5.3, and after dilution with 3 volumes of 0.1 M CH_3COONa at the same pH, the eluent was applied to a column of DE-52 (Whatman) 23 cm high with a diameter of 5 cm. The column was washed with 200–300 mL of 0.1 M CH_3COONa/0.05 M NaCl, pH 5.3, and then with 1800 mL of the same buffer containing 0.08 M NaCl. At this point, the yellow protein band could be seen one-half to two-thirds of the way down the column. The RBP was then eluted by raising the NaCl concentration to 0.11 M. The eluted protein was concentrated by first adding 3 volumes of buffer containing no salt, applying the solution to a short column of DE-52, and eluting with buffer containing 0.2 M NaCl. The concentrated protein solution was dialyzed against 0.05 M ammonium acetate, lyophilized, and redissolved in water and lyophilized 2 more times. The dried powder (yield 0.5–1 g) was stored at 4 °C in a desiccator.

For use, the protein was redissolved in the buffer chosen for each particular experiment, dialyzed against more of the same buffer to ensure complete equilibration, and mixed with the OH· radical scavenger *tert*-butyl alcohol to a concentration of 0.1 M. Concentrations of oxidized RBP were estimated

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[1] Abbreviations: RBP, riboflavin binding protein; $RBPF^-$ and RBPFH, anionic and neutral one-electron-reduced flavin semiquinone forms of the riboflavin binding protein; e_{aq}, hydrated electron; $XSSX^-$, the disulfide–electron adduct; ^0A, ^1A, and ^2A, three protein difference spectra (with oxidized riboflavin binding protein as reference) in the temporal order of observation after reduction of RBP with e_{aq}.

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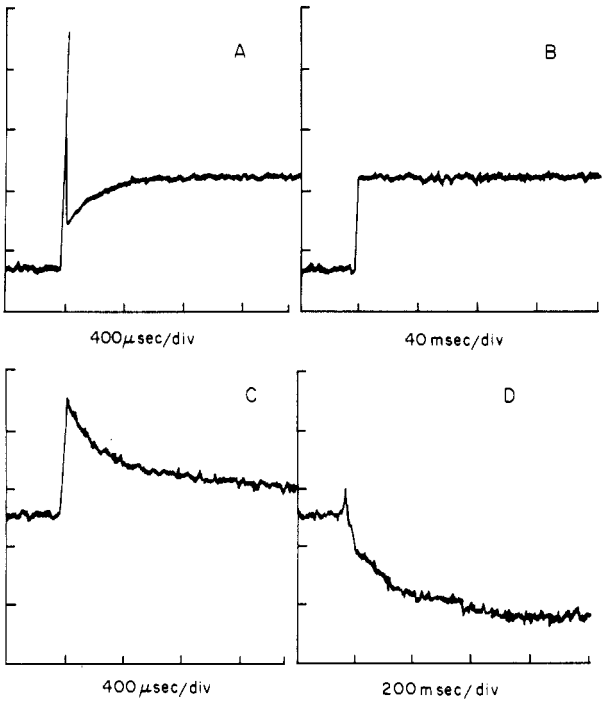


FIGURE 1: RBP reaction with e_{aq}: absorbance changes. The initial concentrations of RBP and hydrated electron were 4.9×10^{-5} M and 0.87×10^{-5} M, respectively, in 0.5 M *tert*-butyl alcohol, 0.002 M borate, and 0.002 M phosphate buffer, pH 9.0, 23 °C. Approximately 20% of the electrons are lost to competing radical–radical recombination and do not react with the protein. Note the differences in the time scales. (A and B) 530 nm, vertical scale is 2% change in transmission per division; (C and D) 410 nm, vertical scale is 2.2% change in transmission per division.

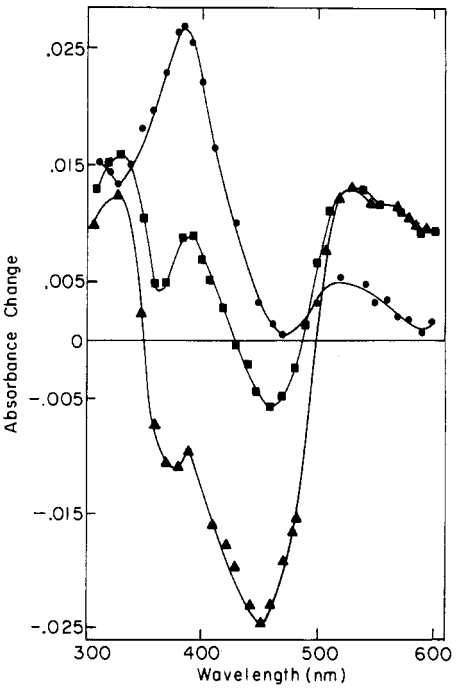


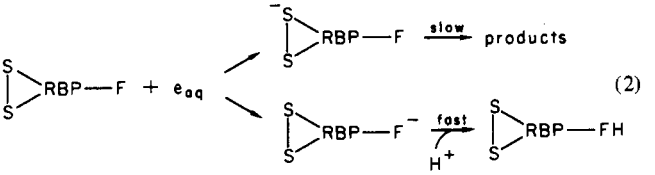
FIGURE 2: Difference spectra after reaction of e_{aq} and RBP. Reaction conditions are the same as given in Figure 1. Difference spectra based on oxidized RBP as reference. (●) Spectrum ^{0}A, reconstructed from the absorbancies observed immediately after the decay of the electron; (■) spectrum ^{1}A, 1 ms after the pulse; (▲) spectrum ^{2}A, 0.7–0.8 s after the pulse.

from the optical density at 455 nm by using a molar absorbance of 13.9×10^3 M^{-1} cm^{-1}. After a degassing with argon, the protein solution was ready for pulse radiolysis. Reagents other than protein were purchased commercially and used as received except for *tert*-butyl alcohol, which was purified by multiple recrystallization. All solutions were prepared with water obtained from a Millipore Q filter.

The computer-controlled pulse radiolysis facility used in this study at the Hebrew University, Jerusalem, employs a Varian V-7715 linear accelerator delivering high-energy (5-MeV, 200-mA) pulsed electron beams of 50–1500-ns duration. For this work, pulse lengths of 1000 ns were used to obtain hydrated electron concentrations of $(7–8) \times 10^{-6}$ M, with a reproducibility over a single experimental series of approximately ±3%. The reaction cell ($0.2 \times 0.3 \times 2.0$ cm) was made of spectrosil with the long axis serving as the optical path. The detection system was constructed with a 150-W xenon arc lamp, mirror optics, a Hilger and Watts D330/331 double-grating monochromater, and an RCA 1P28A or RCA 4840 photomultiplier tube. Appropriate filters were used to minimize photochemical and scattered light effects. Transient transmissions were captured on a Biomation 8100 recorder with suitable electronic sample and hold circuitry or converted directly to digital form for storage on a Nova 1200, the computer that controlled all data gathering, storage, and analysis.

Results and Discussion

From the changes in absorbance with time (Figure 1), we can separate the RBP–hydrated electron reaction into three phases. The first, attachment of the electron to the protein, is seen clearly at 530 nm (Figure 1A) where the immediate, positive absorbance due to e_{aq} decays rapidly to yield a product(s) with positive absorbance. There is a second rapid transition to a form(s) with even greater absorbance at 530 nm and then no further changes (Figure 1B). At 410 nm, the first process, electron attachment, is not seen (Figure 1C) due to the relatively small absorbance of e_{aq} at this wavelength. The product(s) of that attachment has (have) positive absorption. The second process, which occurs with an absorbance increase at 530 nm, is observed as an absorbance decrease at 410 nm, and a slower third process with an absorbance decrease is also seen at this wavelength (Figure 1D). We propose the scheme shown in eq 2 in explanation of the observed results.



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In this reaction scheme there is a minimum of two independent reductions: attachment of the electron either to a disulfide bond of RBP or to the protein-bound flavin. The disulfide–electron adduct then decays slowly, while the anionic flavin semiquinone, $RBPF^-$, is protonated rapidly to the stable neutral species. The evidence and arguments that led us to this proposal follow.

The first observed spectral alteration, presented in Figure 2, spectrum ^{0}A, as a difference spectrum (protein solution before the pulse as reference for ^{0}A, ^{1}A, ^{2}A), immediately follows the loss of e_{aq} from solution. The absorbance increase above 500 nm must be due to the bound flavin, since no other group on the protein could be reduced to a product that absorbs in this region (Klapper & Faraggi, 1979). Thus, attachment of the hydrated electron to the protein results in flavin reduction. This first difference spectrum rapidly gives way to a second, ^{1}A, in which the absorbance at 410 nm is decreased and that at 530 nm increased (Figure 1). This second spectrum decays in turn to the last difference spectrum, ^{2}A,

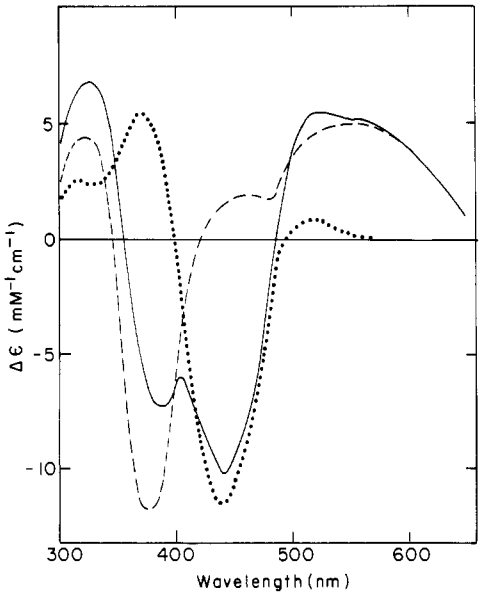


FIGURE 3: Riboflavin difference spectra. Spectra calculated from results of Land & Swallow (1969). (—) Curve A, protonated riboflavin semiquinone/oxidized riboflavin; (···) curve B, anionic riboflavin semiquinone/oxidized riboflavin; (– –) curve C, protonated riboflavin semiquinone/anionic riboflavin semiquinone.

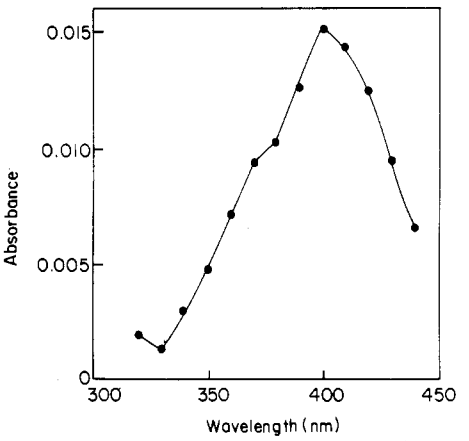


FIGURE 4: Difference–difference spectrum $^1A - {}^2A$. The final difference spectrum of RBPFH (Figure 2, spectrum ^2A) subtracted from the intermediate spectrum collected 1 ms after the pulse (Figure 2, spectrum ^1A). Experimental conditions are those described in Figure 1.

characterized by a decreased absorbance at 410 nm with no change at 530 nm (Figure 1). No further changes were observed over the remaining times of our longest experiments (1–2 s). Because of the temporal separation between the three reaction phases (e.g., at pH 7 with the protein concentrations used, the apparent first-order rate constants associated with the electron decay and the transition of ^0A to ^1A and of ^1A to ^2A were 7×10^5 s^{-1}, 1.6×10^3 s^{-1}, and 15 s^{-1}, respectively), we can obtain reasonable estimates of the three difference spectra ^0A through ^2A (Figure 2).

We begin with a consideration of ^2A (Figure 2, spectrum ^2A), which resembles the difference spectrum between the neutral semiquinone and oxidized forms of riboflavin in aqueous solution [Figure 3, curve A; calculated from Land & Swallow (1969)]. This similarity indicates that a final product of the one-electron reduction of RBP is the neutral semiquinone form of riboflavin bound to the protein (RBPFH). Reduction must involve only one electron, since the e_{aq}/RBP ratio is sufficiently small so that there is little probability of one protein molecule reacting with more than one electron. The semiquinone must be bound to the protein, since free riboflavin semiquinone is not stable (Land & Swallow, 1969) over the times of our experiments. However, RBPFH cannot be the only product of the overall reaction, since we estimate, using the free-riboflavin difference spectrum reported by Land and Swallow, that approximately 25% of the electrons originally bound to protein molecules react to form RBPFH.

The difference spectrum ^1A is identical with ^2A above 510 nm, suggesting that the neutral semiquinone contributes equally to both. But the two spectra are not the same at lower wavelengths (Figure 2, spectra ^1A and ^2A). The nature of this dissimilarity is seen more clearly in the difference of the difference spectra, ^1A–^2A (Figure 4). ^1A contains a component with a peak maximum between 400 and 410 nm, which disappears during the slow decay to the final, stable species. We conclude that the difference spectrum ^1A is due to RBPFH plus at least one other species, which on the basis of the position and relative magnitude (Hoffman & Hayon, 1972; Faraggi et al., 1975) of the peak shown in Figure 4 is a disulfide–electron adduct ($XSSX^-$). $XSSX^-$ is known to be unstable in small model systems, breaking down to the sulfide anion XS^- and the thiyl radical XS·, neither of which would be observed under the conditions of our experiments. The decay rate of $XSSX^-$ in RBP is slower than that in the small linear disulfides (Hoffman & Hayon, 1972) but is comparable to that found with cyclic disulfides (Faraggi et al., 1975). On the basis of reported extinction coefficients of the small-molecule disulfide radicals, we estimate a yield of this adduct on RBP at approximately 25%. Since no more than one electron addition is likely on each protein molecule, the disulfide–electron adduct and the neutral flavin semiquinone do not occur together on the same molecules. For the argument upon which this conclusion is based, see Klapper & Faraggi (1979). Moreover, the disulfide adduct cannot be a precursor of RBPFH, since the identity of the difference spectra ^1A and ^2A above 510 nm indicates that no neutral semiquinone is formed during the decay of $XSSX^-$.

We turn finally to ^0A, the initial difference spectrum after the decay of the hydrated electron (Figure 2, spectrum ^0A). Having argued that $XSSX^-$ contributes to ^1A, it is reasonable to assume that this radical contributes to ^0A as well, since it is known that e_{aq} reduction of disulfide bonds on proteins is a diffusion-limited process [e.g., Bisby et al. (1976) and Faraggi et al. (1978)]. Removing this proposed contribution by subtraction of the $XSSX^-$ spectrum of Figure 4 from ^0A yields a spectrum (Figure 5, curve A) that resembles the difference spectrum between anionic semiquinone and oxidized riboflavin in aqueous solution (Figure 3, curve B). This resemblance suggests that one-electron reduction of RBP produced $XSSX^-$ or $RBPF^-$, which is consistent with the difference–difference spectrum of $^1A - {}^0A$. If RBPFH were a component in ^1A, while $RBPF^-$ were a component in ^0A, and if $XSSX^-$ contributed equally to both, then $^1A - {}^0A$ should be the difference spectrum RBPFH – $RBPF^-$. Comparison of Figures 5, curve B, and 3, curve C, supports this argument. As stated above, the initial electron attachment must yield disulfide radicals and anionic semiquinone flavins on different protein molecules; thus, their formation from e_{aq} is competitive. The reaction scheme presented in eq 2 is consistent with the data and arguments presented in these last three paragraphs.

Before we consider the kinetics associated with this scheme, some comment should be made concerning yields. We have estimated that flavin semiquinone and disulfide radical products (formed on different molecules) together account for approximately 50% of the total e_{aq} attached to the protein.

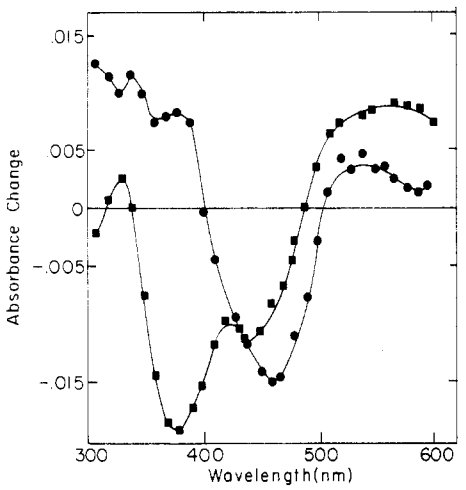


FIGURE 5: Difference–difference spectra. (●) Curve A, the difference spectrum of Figure 4, $^1A - {}^2A$, subtracted from ^0A, the difference spectrum obtained immediately after the electron decay (Figure 2, spectrum ^0A). (■) Curve B, the difference spectrum immediately after the pulse (Figure 2, spectrum ^0A) subtracted from that collected 1 ms after the pulse (Figure 2, spectrum ^1A).

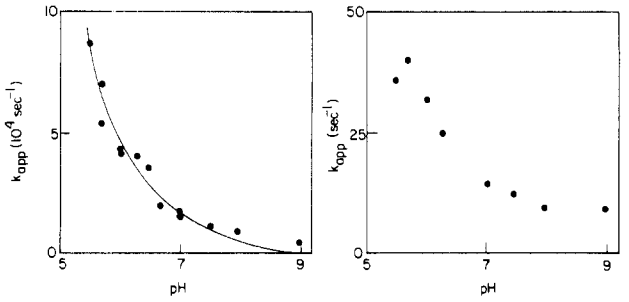
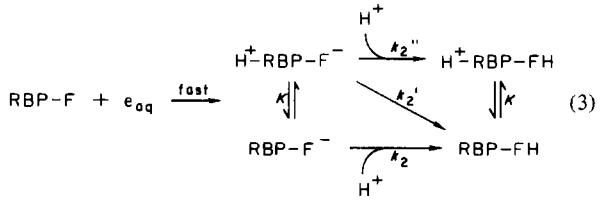


FIGURE 6: Dependence of apparent rate constants on pH. (Right) Decay of $XSSX^-$; (left) protonation of $RBPF^- \rightarrow RBPFH$. The solid line is the best fit calculated on the basis of eq 4. The extracted constants are $k_2K + k_2' = 2.84\ (\pm 0.32) \times 10^4\ s^{-1}$, $k_2'' = 1.82\ (\pm 0.22) \times 10^{10}\ M^{-1}\ s^{-1}$, and $pK = 7.3\ (\pm 0.3)$.

From model studies, we know that, in addition to these two, there are a number of other potential sites for e_{aq} attachment to RBP (Klapper & Faraggi, 1979). These reactions have not yet been detected with any protein due to background interferences, and thus, the unaccounted electrons are "lost" to us at these other sites.

The e_{aq}–RBP reaction with concomitant formation of $XSSX^-$ and $RBPF^-$ was under diffusion control with a rate constant of $1.4 \times 10^{10}\ M^{-1}\ s^{-1}$ at pH 7.0. The subsequent protonation of $RBPF^-$ to RBPFH can be followed above 500 nm (Figure 1A), where $XSSX^-$ does not absorb; and the slower decay of $XSSX^-$ can be monitored near 410 nm (Figure 1D), where the absorbancies of the two protein species, $RBPF^-$ and RBPFH, are similar. The rate constants for both the decay of the disulfide radical and the protonation of the flavin semiquinone are pH dependent (Figure 6). The apparent first-order rate constant for the decay of $XSSX^-$ decreases with pH, which is consistent with the behavior of model compounds (Hoffman & Hayon, 1972; Faraggi et al., 1975).

In the case of the protonation reaction, $RBPF^-$ to RBPFH, the pH variation of the rate constant (Figure 6) rules out a simple, direct combination of $RBPF^-$ with solution protons. For such a case, the computed second-order rate constant, $k_{app}/[H^+]$, should be pH independent, which is not the case. For example, the second-order rate constant calculated from the k_{app} obtained at pH 5.5 was $2.8 \times 10^{10}\ M^{-1}\ s^{-1}$ and that obtained at pH 7.5 was $3.6 \times 10^{11}\ M^{-1}\ s^{-1}$. Not only are the two quite different but also the second would indicate an impossible situation, a reaction faster than diffusion controlled. In other words, the decline of the apparent first-order protonation rate constant with pH is too shallow for protons free in the solvent to be the only source. That the inorganic phosphate present as buffer in the aqueous solution is a proton source is unlikely, since we could not observe a direct relationship between rate and buffer concentration. For example, at pH 7, a 500% increase in the phosphate concentration resulted in a 20% increase of the rate constant. We propose, therefore, that at least some of the protons must come from a group on the protein, either attached covalently or bound tightly, e.g., a protein side chain or bound phosphate. However, since the pH dependence of k_{app} does not display the sigmoidal shape expected were an acidic group on the protein the sole proton donor, we propose that free protons in solution participate as well. In the scheme shown in eq 3, a proton is



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transferred to the anionic semiquinone either directly from solvent (k_2 and k_2'') of via an acid/base group on the protein (k_2').

If one assumes the donor on the protein undergoes a rapid acid/base equilibration, the pH dependence of the apparent rate constant predicted by this scheme is given by

$$k_{app} = \frac{[H^+]}{[H^+] + K}(k_2K + k_2' + k_2''[H^+]) \qquad (4)$$

The nonlinear least-squares fit of the data to eq 4 is shown in Figure 6 (solid line), with the best fit values of K, k_2'', and the composite $k_2K + k_2'$ given in the legend. The magnitude of the best fit second-order rate constant for solvent proton donation, $k_2'' = 1.8 \times 10^{10}\ M^{-1}\ s^{-1}$, is reasonable for a reaction expected to be diffusion controlled. Because of the form of eq 4, a direct estimate of k_2' is not possible. On the reasonable assumption that $k_2 \approx k_2''$, the estimated rate constant for internal proton transfer becomes $3 \times 10^4\ s^{-1}$. These results are consistent with the proposal leading to eq 4. However, we should note that we have no other independent support for the attractive idea of internal proton transfer and that as seen in Figure 6 (left side) the apparent rate constants obtained above pH 7.5 remain too high even for this model. (Though the discrepancy could be explained in terms of proton transfer from a second acid group on the protein, or from water.)

In summary, we conclude from our observations that the one-electron reduction of the flavin moiety in RBP yields, first, the anionic semiquinone, which is then rapidly converted to the stable, neutral semiquinone with half-times ranging from 8 to 90 μs, dependent upon pH. This latter reaction may be assisted by an acid/base group on the protein with a pK_a near 7. We cannot know whether these results have a direct bearing on the reaction mechanisms of flavoenzymes, since RBP is not known to function physiologically either as a redox protein or as an oxidase. Were RBP a reasonable model for flavoenzymes, then the putative role of an acid/base group in the formation of a neutral flavin semiquinone would be particularly intriguing.

Acknowledgments

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Characterization of the *Escherichia coli* X-ray Endonuclease, Endonuclease III†

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ABSTRACT: The X-ray endonuclease endonuclease III of *Escherichia coli* has been purified to apparent homogeneity by using the criterion of sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The most purified fraction shows endonucleolytic activity against apurinic and apyrimidinic (AP) sites and a dose-dependent response to DNA that has been X irradiated, UV irradiated, or treated with OsO_4. The endonuclease also nicks OsO_4-treated DNA that has been subsequently treated with alkali to produce fragmented thymine residues and DNA treated with potassium permanganate. The enzyme does not incise the alkali-labile sites present in DNA X irradiated in vitro in the presence of hydroxyl radical scavengers. The most purified fractions exhibit two distinct activities, an AP endonuclease that cleaves on the 3′ side of the damage leaving a 3′-OH and a 5′-PO_4 and a DNA *N*-glycosylase that recognizes at least two substrates, thymine glycol residues and urea residues. The glycosylase activity is sensitive to *N*-ethylmaleimide while the AP endonuclease is not.

Ionizing radiation produces a variety of damages in DNA including single- and double-strand breaks, alkali-labile damages, and both DNA–DNA and DNA–protein cross-links [see Ward (1975) for review]. The primary criterion for establishing these categories is the particular measurement used to quantitate the damage. Base damages are the preponderant lesion produced (Cerutti, 1975a,b) and are included in all of these categories. However, the quantitation and characterization of base damages are difficult because small numbers of each particular type of damage are present in irradiated DNA, and the instability of some of these damages precludes their measurement by chemical means.

At present, one class of base lesions can be quantitated (Hariharan, 1980), the common radiolysis product of thymine (Scholes & Weiss, 1960), moieties of the 5,6-dihydroxydihydrothymine type (thymine glycols). Thymine glycols have been shown to be lethal lesions in both single-stranded (Hariharan et al., 1977) and double-stranded (Moran et al., 1980) phage transfecting DNAs. Further, *Micrococcus radiodurans* have been shown to specifically release this product into the medium following γ irradiation (Hariharan & Cerutti, 1972). Also, crude extracts of *Escherichia coli* remove thymine glycols from γ-irradiated or OsO_4-treated DNA (Hariharan & Cerutti, 1974). Thus, it is likely that an excision type repair mechanism might function in cells to remove X-ray-induced base damage from DNA.

A number of years ago this laboratory reported that partially purified extracts of *E. coli* were capable of specifically nicking X-irradiated (Strniste & Wallace, 1975) and OsO_4-treated (Armel et al., 1977) DNA; this activity was called the X-ray endonuclease. Subsequently, Radman (1976) reported the purification of an endonuclease from *E. coli*, endonuclease III, that recognized minor photoproducts of UV irradiation, and a similar activity was reported by Gates & Linn (1977b) to nick DNA that was heavily UV irradiated, X irradiated, OsO_4 treated, or depurinated by heat/acid treatment. This latter activity, believed to be endonuclease III, was a byproduct of the purification of endonuclease V (Gates & Linn, 1977a). It has been reported recently (Demple & Linn, 1980) that the enzyme isolated by Gates and Linn has an associated DNA glycosylase specific for thymine glycol residues. Another DNA glycosylase, specific for urea residues in DNA, has been found in 10-fold purified extracts of *E. coli* by Breimer & Lindahl (1980); such urea residues are known products of the radiolysis of thymine residues in DNA (Têoule et al., 1977).

In this paper we report purification of the X-ray endonuclease and show these highly purified preparations to contain

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