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Intramolecular Electron and Proton Transfer in Proteins: CO_2^- Reduction of Riboflavin Binding Protein and Ribonuclease A

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ABSTRACT: The formate radical (CO_2^-) reacts with ribonuclease A to form the cystine disulfide radical as one of the products. CO_2^- reacts with the riboflavin binding protein of chicken egg white with the ultimate product being the neutral flavin semiquinone. Formation of the disulfide radical in ribonuclease is slower than the reaction between protein and CO_2^-; formation of the flavin semiquinone in the riboflavin binding protein is slower than the protein–CO_2^- reaction. We conclude for both proteins that CO_2^- must reduce an as yet unidentified group or groups, which in turn reduce(s) the disulfide of RNase or the flavin of riboflavin binding protein. This conclusion is supported in the case of ribonuclease by the observation of a transient, broad absorption band centered between 350 and 370 nm. The CO_2^--initiated reductions of the disulfide in ribonuclease and the flavin in the riboflavin binding protein are mixed first- and second-order processes. We propose that the transfer of an electron from the unknown intermediate(s) to the final product involves both inter- and intramolecular paths between groups that may not be in van der Waals contact. With the hydrated electron, in contrast to CO_2^-, as reductant of the riboflavin binding protein, the anionic semiquinone is observed as an intermediate. The anionic semiquinone is then rapidly protonated, yielding the stable neutral semiquinone. From the reaction kinetics and protein concentration dependence, we conclude that a group or groups on the protein donate(s) a proton to the anionic semiquinone by both inter- and intramolecular paths.

The proposal that electrons can migrate or transfer over long distances in proteins has been made a number of times, and for a variety of reasons [e.g., Winfield (1965), Grossweiner (1976), and Klapper & Faraggi (1979)]. Not only does the existence of such transfer have significance for the understanding of physiological redox reactions, but we have also suggested (Steiner et al., 1985) that the detection and study of protein intramolecular electron transfer may yield information about internal electrostatic properties of proteins. There have been a number of recent reports on electron transfer between groups that are not in van der Waals contact in small model systems [e.g., Prutz et al. (1981), Guar et al. (1983), Calcaterra et al. (1983), and Isied & Vassilian (1984)]. Two groups (Winkler et al., 1982; Isied et al., 1984)

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have proposed a 15-Å intramolecular transfer in cytochrome *c* with a rate constant of 20–50 s^{-1}. Prütz and co-workers (Prütz et al., 1980) have observed transfer from tyrosine to tryptophan over unknown distances in a number of proteins. Because of the significance of long-range electron transfer in proteins, we have also been searching for its existence. In this paper we describe our results with the two radical reductants, CO_2^- and the hydrated electron (e^-_{aq}),[1] reacting with the riboflavin binding protein from chicken egg white (RBP) and ribonuclease A (RNase). We have presented the RBP results elsewhere in a preliminary form (Faraggi & Klapper, 1984).

Materials and Methods

Pulse radiolysis experiments were performed in the linear accelerator facility of The Ohio State University (Felix et al., 1967). The primary radicals were produced with a short pulse (0.1–0.5 μs) of 3.5-MeV electrons impinging on an aqueous protein solution. When e^-_{aq} was to be the reductant, the solution contained phosphate buffer (5 mM) and 2-methyl-2-propanol (0.1 M) to scavenge hydroxyl radical and was purged for approximately 30 min with argon to remove oxygen. When CO_2^- was to be the radical reductant, the 2-methyl-2-propanol was replaced with sodium formate (0.1 M), and the solution was deaerated with N_2O (first passed through three purging towers containing 0.1 M vanadyl sulfate/sulfuric acid/amalgamated zinc to remove contaminating oxygen and then through three towers of water; Meites & Meites, 1948) for approximately 30 min. The N_2O and formate in the reaction mixture ensures that all primary radicals produced in the pulse are converted immediately to CO_2^- by the reactions

$$e^-_{aq} + N_2O \xrightarrow{H^+} OH + N_2$$

$$OH + HCOO^- \rightarrow H_2O + CO_2^-$$

$$H + HCOO^- \rightarrow H_2 + CO_2^-$$

The concentrations of e^-_{aq} and CO_2^- were in the range of 1–10 μM as determined by thiocyanate dosimetry (Fielden, 1982). The protein to radical concentration ratios were generally kept at 10 or higher to minimize multiple radical reactions with one protein molecule.

After exposure of the sample solution to a single pulse from the accelerator, the time course of the reaction was followed by the absorbance changes at one wavelength. A solution was used only once in order to avoid additional reactions with reduction products that would have accumulated after the previous pulse. A spectral time dependence was, when needed, reconstructed from individual experiments at different wavelenghts.

RBP was isolated from chicken egg whites as described elsewhere (Klapper & Faraggi, 1983). Chromatographically purified RNase A was purchased from Worthington Biochemicals (Freehold, NJ). All other reagents were purchased commercially and used without further purification, except for 2-methyl-2-propanol, which was recrystallized 3 times. Water was purified through a Millipore Q system.

Results and Discussion

Riboflavin Binding Protein. (A) Hydrated Electron Reaction. The hydrated electron (e^-_{aq}) reacts with the egg white riboflavin binding protein (RBP), at or near diffusion control (Klapper & Faraggi, 1983). The two products observed initially are the disulfide/electron adduct on the protein ($RBP–RSSR^-$) and the one electron reduced, protein-bound anionic flavin semiquinone ($RBP–Fl^-$). Because of the large protein to e^-_{aq} ratio, only a small fraction of the protein molecules are reduced, and on the basis of a statistical argument, the two radical products do not occur together on the same molecules (Klapper & Faraggi, 1979). The identifications of these two species are based on spectral evidence, as discussed elsewhere (Klapper & Faraggi, 1983), but their formation can be detected at 350 and 540 nm as absorbance increases that parallel the decay of e^-_{aq}. The protonation of $RBP–Fl^-$ to form the neutral semiquinone (RBP–FlH) is then observed as a further absorbance increase at 540 nm together with an absorbance decrease at 350 nm (Figure 1; k_{app} = 4.8 × 10^3 s^{-1} at pH 9.0 and 5.6 × 10^{-5} M protein). There is an additional, slower absorbance decline at 350 nm (k_{app} = 9.7 s^{-1}) due to the loss of the disulfide radical. On the basis of the more extensive data presented in our previous paper (Klapper & Faraggi, 1983), we proposed that the observed kinetics were most simply explained with the scheme

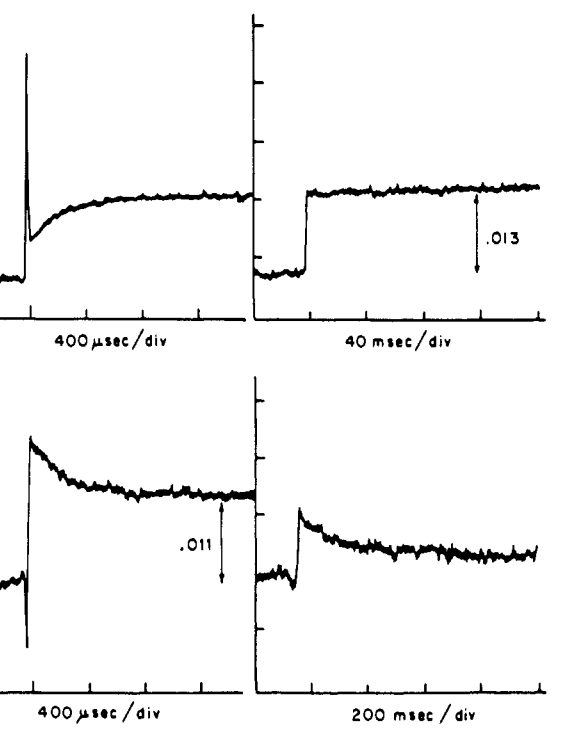
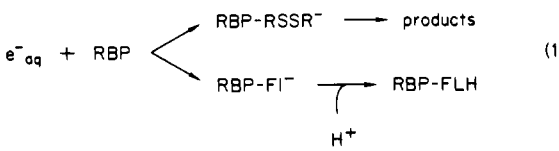


FIGURE 1: Absorbance changes in the reaction of e^-_{aq} with RBP at 540 (top) and 350 nm (bottom). The conditions were identical for all panels: RBP concentration = 5.6 × 10^{-5} M, e^-_{aq} concentration = 7.9 × 10^{-6} M, and pH 9.0. The spike in the top-left panel is due to the rapid formation and reaction of e^-_{aq} itself.



(1)

While the products of the $RBP–RSSR^-$ decay are not known, it has been proposed that $RSSR^-$ in small linear compounds is cleaved to the thiyl radical plus thiolate ion (Hoffman & Hayon, 1972).

Because the slower of the absorbance increases at 540 nm is due entirely to the protonation of the anionic semiquinone, the kinetics of this reaction are easily studied. Although the formation of the protonated semiquinone obeys a first-order

[1] Abbreviations: RNase, pancreatic ribonuclease A; RBP, riboflavin binding protein; $RBP–Fl^-$, anionic semiquinone form of RBP; RBP–FlH, protonated semiquinone form of RBP; e^-_{aq}, hydrated electron; CO_2^-, formate radical; $RSSR^-$, one electron reduced disulfide bond, the disulfide radical anion; OH, hydroxyl radical; H, monoatomic hydrogen.

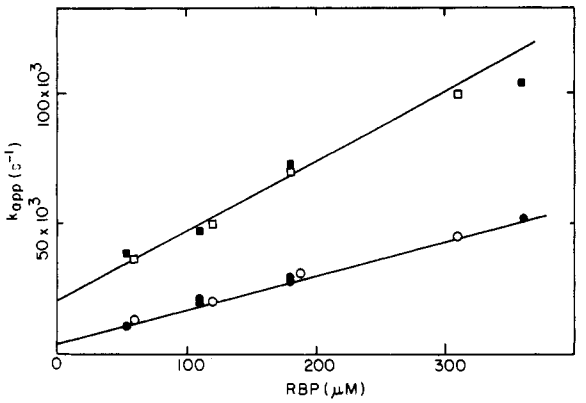


FIGURE 2: Dependence of anionic semiquinone protonation rate constant on pH, ionic strength, and RBP concentrations: (●) pH 9; (○) pH 9, 0.1 M NaCl, $k_{intercept}$ = 3.6 (0.6) × 10^3 s^{-1}, k_{slope} = 14.3 (0.6) × 10^7 M^{-1} s^{-1}; (■) pH 5.9; (□) pH 5.9, 0.1 M NaCl, $k_{intercept}$ = 20 (2) × 10^3 s^{-1}, k_{slope} = 26 (2) × 10^7 M^{-1} s^{-1}.

rate law, the apparent rate constant for this process is linearly dependent, with an extrapolated non-zero intercept, on RBP concentration (Figure 2). This suggests that there are both first- and second-order components to the reaction. One explanation for such results might be based on the single-step equilibrium

$$\text{RBP–Fl}^- \text{ H}^+ \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{RBP–FlH} \qquad (2)$$

$$K_a = k_{-1}/k_1$$

$$k_{app} = k_1([H^+] + K_a)$$

However, this simple scheme predicts an apparent first-order rate constant that is independent of the RBP concentration and so is inconsistent with the experimental results. We must construct a more complex scheme, but one that does not include eq 2 to account for the first-order process required by the data of Figure 2. That no explanation is valid if it involves the solvent as proton donor (eq 2) is based on the following contradiction. Assume protonation is diffusion controlled, in order to assign the maximum possible value to k_1 of 4 × 10^{10} M^{-1} s^{-1}. Then, taking the value of k_{app} at pH 9.0 as 3.6 × 10^3 s^{-1} from the intercept of the curve in Figure 2, we can calculate a pK_a of 7 for the semiquinone from the expression for k_{app} in eq 2. This value, which would be even lower had we assigned a smaller protonation rate constant, is far too low since RBP–FlH is still protonated at pH 9.0 (Figure 3). For the same calculation at lower pH, the discrepancy is even worse, although below pH 5 hydronium ion may serve as a proton donor (Klapper & Faraggi, 1983). Nor can the solution buffer act as proton donor, since we have already shown that the apparent protonation rate constant is insensitive to the buffer concentration (Klapper & Faraggi, 1983).

A minimal scheme consistent with the experimental data presented here posits a protein acid/base group that serves as proton donor in both intra and intermolecular reactions.

$$\text{HA–RBP–Fl}^- \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} {}^-\text{A–RBP–FlH}$$

$$\text{HA–RBP–Fl}^- + \text{HA–RBP} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} {}^-\text{A–RBP} + \text{HA–RBP–FlH} \qquad (3)$$

$$k_{app} = \frac{k_1[H^+] + k_{-1}K_a'}{K_a' + [H^+]} + \frac{k_2[H^+] + k_{-2}K_a'[\text{RBP}]}{K_a' + [H^+]}$$

in which K_a', the acid dissociation constant for the protein group –AH, is assumed to be unaffected by the protonation

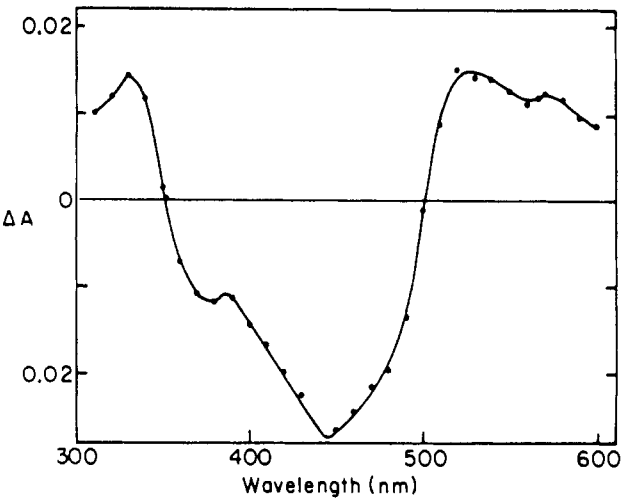


FIGURE 3: Difference spectrum, RBP–FlH – RBP. Spectrum obtained 500 ms after the end of the RBP–CO_2^- reaction. RBP concentration = 5.4 × 10^{-5} M; CO_2^- concentration = 3.5 × 10^{-6} M; pH 9.0.

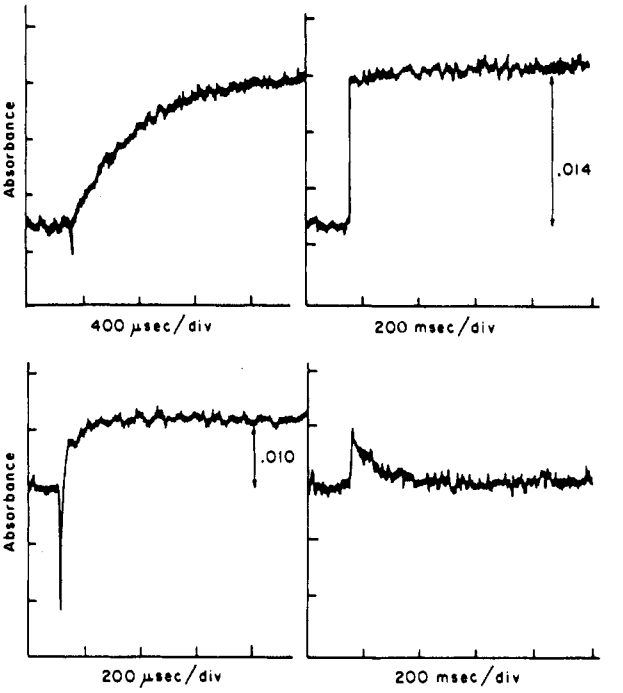


FIGURE 4: Absorbance changes in the reaction of CO_2^- with RBP at 540 (top) and 352 nm (bottom). The experimental conditions were identical for all panels and are given in the legend of Figure 3.

or oxidation state of the flavin semiquinone.[2] We also assume that direct protonation from the solvent is negligible at these higher pHs due to the low hydronium ion concentration (see above argument) and that the concentration of RBP is in sufficient excess to remain constant during the reaction so that HA–RBP is the predominant proton donor (RBP–Fl^- and RBP–FlH are ca. 10% of the total protein concentration even at the lowest experimental protein to e^-_{aq} ratios.) It should be noted that both the reactions in eq 3 are required, since the first is RBP independent in both directions and the second is RBP dependent in both directions.

(B) CO_2^- Reaction. Protein-bound disulfide radical and protonated flavin semiquinone are also products in the reaction

[2] We note that while this minimal scheme is consistent with the data, we do not wish to imply that the acid dissociation constant for the protein group –AH is in fact identical in all protonation and oxidation states of the flavin. There are insufficient data to address this point.

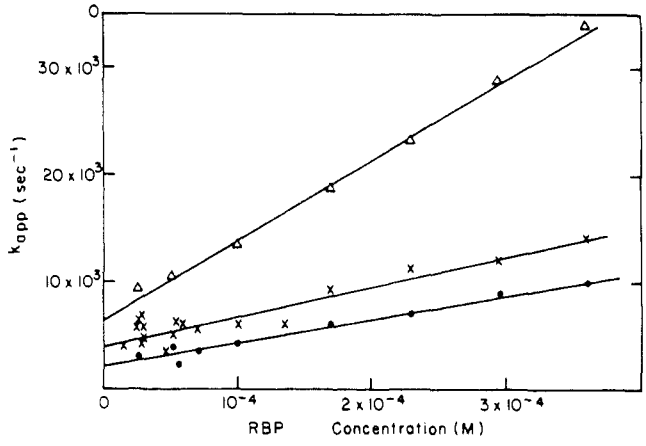


FIGURE 5: Dependence of flavin reduction rate constant on pH and RBP concentration. Experimental conditions were those of Figure 3 except for pH and RBP concentration variations: (●) pH 9.0, $k_{intercept}$ = 2.10 (0.32) × 10^3 s^{-1}, k_{slope} = 2.24 (0.19) × 10^7 M^{-1} s^{-1}; (×) pH 7.0, $k_{intercept}$ = 4.16 (0.31) × 10^3 s^{-1}, k_{slope} = 2.63 (0.34) × 10^7 M^{-1} s^{-1}; (△) pH 5.2, $k_{intercept}$ = 6.98 (0.37) × 10^3 s^{-1}, k_{slope} = 7.01 (0.26) × 10^7 M^{-1} s^{-1}.

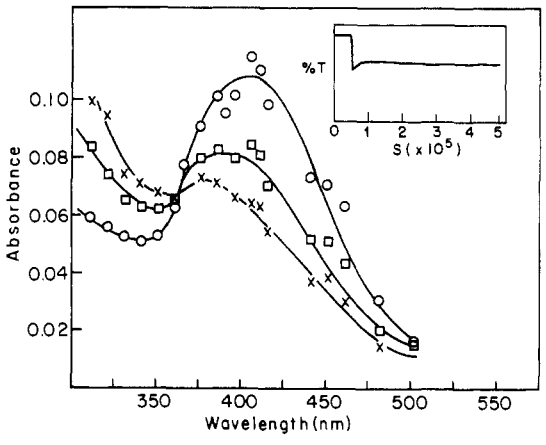


FIGURE 6: Difference spectra on the reaction of CO_2^- with RNase A. The reaction solution contained 3.4 × 10^{-4} M RNase, 0.1 M sodium formate, 5 mM phosphate buffer, pH 7.4, and CO_2^- (1.2 × 10^{-5} M). The deaerated solution was saturated with N_2O. Spectra were taken 10 (×), 20 (□), and 110 μs (○) after the pulse. (Inset) Trace of transmission charge with time measured at the isosbestic point, 370 nm.

of RBP with CO_2^-. The spectrum of the stable RBP–FlH measured after the decay of the disulfide radical is presented in Figure 3. However, in contradistinction with the e^-_{aq} reaction, we find no spectral evidence for either RBP–Fl^- or the flavin protonation reaction. As seen in Figure 4, there is a single first-order absorbance increase at 540 nm with no initial burst due to RBP–Fl^-, and there is only one first-order decay at 352 nm (the isosbestic point between RBP and RBP–FlH) with a rate constant (k_{app} = 9.7 s^{-1}) identical with that for the decay of protein-bound disulfide radical in the e^-_{aq} + RBP reaction. As an additional wrinkle, inspection of Figure 4 reveals a rate of disulfide radical formation (k_{app} = 1.7 × 10^4 s^{-1}) measured at 342 nm that is faster (approximately 7-fold) than the rate of neutral semiquinone formation (k_{app} = 2.3 × 10^3 s^{-1}) measured at 540 nm. Thus, 90% of the disulfide reduction is completed in the same time that only 25–30% of the flavin reaction has occurred. (The approximate yields of the two products relative to the amount of CO_2^- reacted with the protein are 35% RBP–$RSSR^-$ and 40% RBP–FlH). Were CO_2^- reducing both protein disulfide and flavin directly, then the two reactions would have to have the same apparent rate constant.[3] On the contrary, these results require that the CO_2^- reaction with RBP be at least as fast as $RSSR^-$ formation and must produce on the protein an intermediate that serves as the electron donor in the slower flavin reduction. Moreover, the disulfide radical cannot be that intermedate since the rate constant for decay of RBP–$RSSR^-$ is 240-fold smaller than the rate constant for RBP–FlH formation, while the yields of the two products are approximately equal. Thus, we propose the following reaction scheme:

$$CO_2^- + \text{RBP} \begin{cases} \xrightarrow{} \text{RBP-RSSR}^- \xrightarrow{} \text{products} \\ \xrightarrow{} \text{•X-RBP} \xrightarrow{} \text{RBP-FlH} \end{cases} \quad (4)$$

At present we have not identified the presumed radical intermediate •X–RBP.

The first-order process seen at 540 nm in the CO_2^- reaction (Figure 4) is the reduction of flavin to form the neutral RBP–FlH. This is distinctly different than the first-order process seen at 540 nm in the e^-_{aq} reaction (Figure 1), in which the anionic semiquinone is protonated to the same stable product, RBP–FlH. The two processes do, however, share a common trait—the apparent first-order flavin reduction in the CO_2^- reaction also increases linearly with RBP concentration with a linear extrapolation to a finite value on the ordinate (Figure 5).

The protonation equilibrium of the flavin semiquinone involves the N-5 position. Since the pK of this nitrogen in oxidized flavin is below 1 (Land & Swallow, 1969), it is not surprising to find the anionic semiquinone as an intermediate in the e^-_{aq} reduction of RBP. But RBP–Fl^- is not seen with CO_2^- as reductant. Rate constants for RBP–FlH formation in the CO_2^- reaction are presented in Figure 5. On comparison of the results in Figures 2 and 5, we see that the rate constants for protonation of RBP–Fl^- in the e^-_{aq} reaction are faster than those for formation of RBP–FlH in the CO_2^- reaction. We conclude that RBP–Fl^- is most probably an intermediate in the CO_2^- reaction but that it does not accumulate in sufficient amounts for spectral detection, because electron transfer from the unknown intermediate to the flavin is rate limiting. But with this explanation, the protein concentration dependence of the flavin reduction (Figure 5) cannot be explained by the mixed-order protonation scheme of eq 3. The simplest electron-transfer scheme one can propose as the basis of the observed results is

$$\begin{array}{l} \text{•X-RBP} \xrightarrow{H^+} \text{RBP-FlH} \\ \text{•X-RBP} + \text{RBP-Fl} \xrightarrow{H^+} \text{RBP} + \text{RBP-FlH} \end{array} \quad (5)$$

In analogy with the similar argument given above, neither the intramolecular nor the intermolecular reaction of eq 5 can alone account for the observed concentration dependence; the first is independent of RBP concentration in both directions, and the second is dependent on RBP concentration in both directions. But with the two reactions operating in parallel, the results of Figure 5 would be expected.

Ribonuclease–CO_2^- Reaction. The kinetic interpretation of the RBP flavin reduction by CO_2^- suggests that electrons are transferred to the flavin group from an as yet unknown

[3] This would be true irrespective of whether both reduction products were on the same or on different molecules or whether one or the other reaction involved a contaminant—unlikely in the face of the high overall yield.

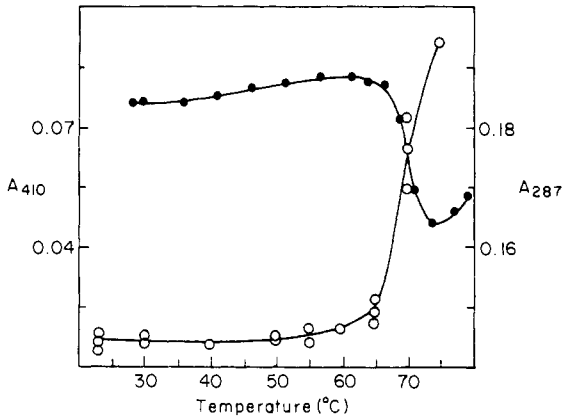


FIGURE 7: RNase melting measured by UV absorbance changes and electron/disulfide adduct yield. Pulse radiolysis conditions were similar to those described in the legend of Figure 6, except the pH was 7.0. Protein absorbance measurements were conducted separately in a UV–visible spectrophotometer with the only difference that of RNase concentration being 34 μM. (○) Temperature dependence of $RSSR^-$ yield as measured by absorbance change at 410 nm obtained at 0.5 ms after the pulse. (●) Protein denaturation measured by absorbance change at 287 nm.

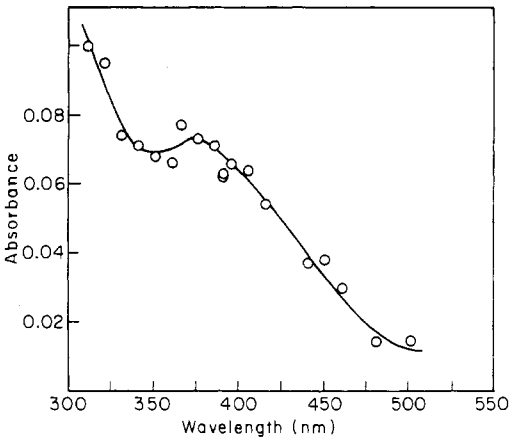


FIGURE 8: Transient absorption spectrum of CO_2^- at pH 7.2. The reaction solution was deaerated and saturated with N_2O and contained 0.1 M sodium formate, 0.005 M phosphate, pH 7.2, and 51 μM CO_2^-.

intermediate in either an intra- or intermolecular process. We have found a striking similarity in the reduction of RNase by CO_2^-. Adams and co-workers (Adams et al., 1972) have reported that while e^-_{aq} reduces the disulfides of RNase, CO_2^- does not unless the protein is first denatured. We were, therefore, surprised to find formation of a "410"-nm band, the characteristic signature of $RSSR^-$ (Figure 6), in our initial studies of this reaction. The $RSSR^-$ absorption band cannot have come from reaction with a contaminant, since the yields, based on the extinction coefficient of the lipoic acid radical (Faraggi et al., 1975), are between 10% and 20% depending on the RNase concentration and are reproducible between different RNase batches. We, therefore, investigated the magnitude of the "410"-nm band after heat denaturation of the enzyme. As seen in Figure 7, the yield of $RSSR^-$ in the CO_2^- reaction increases sharply above 65 °C at pH 7.0. In the same figure we have plotted the protein UV absorbance at 287 nm as a function of temperature. These latter results, obtained in a separate experiment under solution conditions closely similar to those of the pulse radiolysis experiment, show an absorbance decline that reflects protein denaturation (Hermans & Scheraga, 1961). The coincidence of the two curves clearly indicates that the yield of the protein-bound disulfide radical does increase with protein denaturation, in agreement with the observation of Adams and co-workers. The most reasonable explanation is that the majority of RNase disulfide bonds are not accessible to the large, negatively charged CO_2^- until the protein is denatured. The results also rule out the possibility that the small amount of $RSSR^-$ formation at room temperature is due to a small fraction of denatured protein in equilibrium with native enzyme, since the melting of RNase begins at a much higher temperature. While Adams and co-workers have suggested no disulfide reduction at room temperature, there may be no contradiction between their results and our own. An inspection of Figure 6 in their publication suggests that a small absorbance at 410 nm is seen in the first pulse of CO_2^-. That initial absorbance change is dwarfed by the much larger change observed after denaturation in both their and our data.

Returning to Figure 6, we see not only the formation of the "410"-nm band but an earlier absorbing species with a broad maximum between 350 and 370 nm. The decay of this absorbance band is coincident with the rise of the disulfide radical absorbance, and in the transition there is an isosbestic point at 370 nm. Thus, there appears to be an intermediate along the path to $RSSR^-$ formation. Our first concern was that this apparent intermediate might be CO_2^- itself, which has a weak band in this region in addition to the known intense band lower in the ultraviolet (Buxton & Sellers, 1973). The near-UV shoulder in the absorption of CO_2^- is shown in Figure 8. However, the intermediate absorption band seen in the RNase reaction cannot be due to CO_2^-, a conclusion based on the following experiment. When a compound Z that also reacts with CO_2^- is added to the protein solution, the apparent rate constant for the loss of CO_2^- is given by $k_{app} = k_P[\text{RNase}] + k_Z[Z]$, and is identical with the apparent rate constants for both RNase and Z reduction. The primary rate constants, k_P and k_z, can be obtained in one of two ways. On varying the protein concentration k_{app} will increase linearly with a slope equal to k_P and an intercept equal to $k_Z[Z]$. Alternately, the yield of Z·, the reduced competitor, will decrease with increasing protein concentration on the basis of the relationship

$$A_0/A = 1 + ([\text{RNase}]/[Z])k_Z/k_P \qquad (6)$$

With an independent measurement of k_Z, the constant for the reaction between CO_2^- and competitor Z, k_P can be calculated from the slope of the observed A_0/A plotted vs [RNase]/[Z], where A_0 and A are the absorbance yield ratios at 0 and finite RNase concentrations, respectively. We chose as our competitor lipoic acid, which also reacts with CO_2^- to produce $RSSR^-$ (Hoffman & Hayon, 1972; Faraggi et al., 1975). Although both lipoate and RNase disulfides are reduced by CO_2^-, the lipoate reaction is so much faster that at short times after the pulse the observed absorbance of the "410"-nm band is due almost entirely to the lipoate disulfide radical. As expected from eq 6, a plot of A_0/A vs. [RNase]/[Z] obtained from the competition experiment was a straight line with an intercept close to 1.0. From the slope of this plot and the rate constant (4.1×10^8 M^{-1} s^{-1}, obtained in a separate experiment) for the CO_2^- reduction of lipoate, we calculated that the rate constant for the reaction of RNase and CO_2^- at room temperature and pH 7.3 is 3.0×10^8 M^{-1} s^{-1}, a value approximately 10 times greater (based on the computation of a pseudo-first-order constant) than that measured for reduction of the disulfide on the protein. Hence, CO_2^- is largely gone from solution before the transient protein species is converted to RNase($RSSR^-$), and the broad absorbance band centered between 360 and 370 nm cannot be ascribed to CO_2^-.

We conclude that, as in the case of RBP, CO_2^- reduces RNase at some still unknown site[4] to form an intermediate

that then reduces one or more of the protein disulfides. The rate of this second electron transfer can be measured directly from the absorbance increase of the "410"-nm band, and we were able to determine the dependence of the rate constant on the protein concentration. In analogy with the RBP results, we obtained a linear dependence on concentration with a non-zero intercept. (The conditions of the reaction were similar to those described in the legend of Figure 6: $k_{intercept}$, 4.1×10^3 s^{-1}; k_{slope}, 6.8×10^7 M^{-1} s^{-1}; pH 7.6.) Once again, we can rule out a simple one-step equilibrium: for a single intermolecular transfer step the apparent rate constant would be simple second order, linear dependence on the protein (in large excess) concentration with an intercept at the origin; for a single intramolecular transfer step the apparent rate constant would be concentration independent. With RNase, unlike RBP, we need not account for a protonation reaction, since the disulfide radical product is unprotonated, and we conclude that the transfer of an electron from the unknown intermediate to a protein cysteine is a mixed inter- and intramolecular process.

The conclusion we wish to emphasize from the results presented here is that after CO_2^- reduction of both RBP and RNase there is a mechanism whereby an electron can move internally from an as yet unidentified radical(s) on the protein to a second group, flavin in the case of RBP and one or more disulfide bonds in the case of RNase. While we know little of the initial reduction site or sites other than the absorbance of a spectral intermediate with RNase, a comparison of the rate constants extracted from the data of Figure 5, and presented in the paragraph immediately preceding this one, reveals a striking similarity between the first- and second-order rate processes obtained for the two proteins. This suggests, but only tentatively, that the unknown group is the same in both. Since we do not know that initial reduction site(s), and in the case of RBP since we have no crystal structure with which to work, we can say little about the distance over which the intramolecular transfer must occur. However, it is likely that CO_2^-, a large negatively charged species, reacting as rapidly as it does with both these proteins, cannot "diffuse" into the protein interior and must reduce a site on the protein surface. Moreover, we know that CO_2^- does not react directly with the RNase disulfides, most likely because the disulfides are inaccessible to the radical until the protein is denatured. Hence, in RNase at least, the electron transfer must be from a surface-accessible group to a disulfide(s) that is (are) probably inaccessible to CO_2^-. This suggests migration between two sites that are not in van der Waals contact. A similar argument can be made for RBP, since the kinetic evidence cited here suggests that CO_2^- cannot reach the protein's flavin group, which may be largely buried within the protein matrix in analogy with flavodoxin (Mayhew & Ludwig, 1975).

And finally, a technical detail: in both the RNase and RBP reactions the concentration dependence of the electron-transfer reaction is sufficiently small that it will be overlooked if the protein concentration range studied is not sufficiently large. At first glance the observation of a second-order process in an attempt to show intramolecular electron transfer may appear to be an unwanted complication. However, an intermolecular process does not contradict the conclusion of internal electron migration, and the results we have obtained rule out a first-order reaction dependence due either to a slower required protein conformational change or to an initial complex between CO_2^- and protein. Thus, an effort should be made to search for a second-order component in any reaction that may involve internal electron migration.

In summary, we have shown that formation of the one electron reduced flavin semiquinone in RBP or of the disulfide radical in RNase is slower than the reaction of CO_2^-, the initial reductant, with the protein. This imposes the kinetic requirement for an intermediate radical on the protein. In both cases the intermediate is unidentified, although it has been observed spectrally in RNase. From the protein concentration dependence of the rate constants we conclude that the electron transfers from intermediate(s) to flavin or disulfide contain a first-order component that strongly suggests an intramolecular process in both proteins. In a more speculative vein we suggest that the transfers occur from protein surface to CO_2^--inaccessible sites and thus over distances greater than van der Waals contact. These, and the results of other authors described in the introduction, suggest that physiological transfer of an electron from a donor site on one protein to an acceptor site on a second protein need not require direct contact between the two redox centers.

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Registry No. RNase, 9001-99-4; RBP-Fl$^-$, 35919-91-6; RBP-FlH, 83-88-5; CO_2^-, 85540-96-1.

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[4] While we do not know the nature of this site, we feel it cannot be a histidine residue for two reasons. We have been unable to detect reduction of imidazole by CO_2^- in model studies, and the hydrated electron shows little or no reactivity toward histidine in RNase above pH 7.

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Quantitative Characterization of the Binding of Plasminogen to Intact Fibrin Clots, Lysine–Sepharose, and Fibrin Cleaved by Plasmin†

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ABSTRACT: The binding of human Glu- and Lys-plasminogens to intact fibrin clots, to lysine–Sepharose, and to fibrin cleaved by plasmin was quantitatively characterized. On intact fibrin clots, there was one strong binding site for Glu-plasminogen with a dissociation constant, K_d, of 25 μM and one strong binding site for Lys-plasminogen with a K_d of 7.9 μM. In both cases, the number of plasminogen binding sites per fibrin monomer was 1. Also, a much weaker binding site for Glu-plasminogen was observed with a K_d of about 350 μM. Limited digestion of fibrin by plasmin created additional binding sites for plasminogen with K_d values similar to the binding of plasminogen to lysine–Sepharose. This was predictable given the observations that plasminogen binds to lysine–Sepharose and can be eluted with ϵ-aminocaproic acid [Deutsch, D. G., & Mertz, E. T. (1970) *Science (Washington, D.C.) 170*, 1095–1096] and that plasmin preferentially cleaves fibrin at the carboxy side of lysyl residues [Weinstein, M. J., & Doolittle, R. F. (1972) *Biochim. Biophys. Acta 258*, 577–590], because the structures of the lysyl moiety in lysine–Sepharose and of ϵ-aminocaproic acid are identical with the structure of a COOH-terminal lysyl residue created by plasmin cleavage of fibrin. The K_d for the binding of Glu-plasminogen to lysine–Sepharose was 43 μM and for fibrin partially cleaved by plasmin 48 μM. The K_d for the binding of Lys-plasminogen to lysine–Sepharose was 30 μM. With fibrin partially cleaved by plasmin, there were two types of binding sites for Lys-plasminogen, one with a K_d of 7.6 μM and the other with a K_d of 44 μM. Plasmin-created plasminogen binding sites may be physiologically relevant as a mechanism for accelerating clot destruction and because fragments of fibrin and other proteins created by plasmin cleavage at lysyl residues would be targeted for destruction anywhere in the circulatory system. Fibrin was shown to be a positive regulator of the activation of plasminogen by human urokinase. After a lag period, presumably due to impedance of diffusion by the dense, fibrous matrix of the clot, the rate of activation of Glu-plasminogen rapidly accelerated in the presence of fibrin, compared to in its absence or in the presence of fibrinogen.

The role of fibrin in regulating its own destruction is not clear. The enzyme system involved consists of the serum zymogen plasminogen which upon cleavage of a single arginyl–valyl bond by a plasminogen activator becomes the potent protease plasmin that dissolves fibrin clots. Some theories for the regulation of fibrinolytic activity by fibrin stress the importance of plasminogen binding to fibrin (Alkjaersig et al., 1959), of plasminogen activators binding to fibrin (Chesterman et al., 1972), and of the protective effect of fibrin on the fibrinolytic enzymes from attack by protease inhibitors (Wiman & Collen, 1978).

Quantitative studies of the binding of plasminogen to fibrin were first reported by Thorsen (1975). Native or Glu-plasminogen,[1] which is a single polypeptide chain comprised of 790 amino acids including an NH_2-terminal glutamic acid (Wallén & Wiman, 1972), exhibited a modest affinity for fibrin. Lys-plasminogen, a 714 amino acid variant with an NH_2-terminal lysine created by plasmin cleavage of the lysyl–lysyl bond at position 76–77 from the NH_2-terminus of Glu-plasminogen (Robbins et al., 1967; Wallén & Wiman, 1972), exhibited a stronger affinity for fibrin. The interaction between these two forms of plasminogen and fibrin is mediated through lysine binding sites on plasminogen, because only fragments of plasminogen that bind to lysine–Sepharose bind to fibrin, and because binding of plasminogen to fibrin can be abolished by the presence of certain α,ω-amino acids (Wiman & Wallén, 1977).

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[1] Abbreviations: FMGB, 3′-(4-guanidinobenzoyloxy)-6′-hydroxyspiro[isobenzofuran-1(3*H*),9′-[9*H*]xanthen]-3-one; P_i/NaCl, phosphate-buffered saline; Glu-plasminogen, native plasminogen with an NH_2-terminal glutamic acid; Lys-plasminogen, plasminogen with an NH_2-terminal lysine that is produced by plasmin cleavage of the lysyl–lysyl bond at position 76–77 of Glu-plasminogen; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

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