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KINETICS OF SOME ELECTRON-TRANSFER REACTIONS IN BIOLOGICAL PHOTOSYSTEMS

I. PULSE RADIOLYSIS STUDY OF SPINACH FERREDOXIN REDUCTION BY THE HYDRATED ELECTRON AND CO_2^- RADICAL

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The reduction of spinach ferredoxin by the CO_2^- radical and the hydrated electron (e_{aq}^-) has been studied by pulse radiolysis in the pH range between 5.05 and 9.67. The reduction of oxidized spinach ferredoxin by both CO_2^- and e_{aq}^- was found to be essentially quantitative. The CO_2^- radical reduces spinach ferredoxin by a single second-order process at a rate $k_5 = (6.2 \pm 0.6) \cdot 10^7\ M^{-1} \cdot s^{-1}$. Reduction by e_{aq}^- follows a biphasic pathway. The first phase obeys second-order kinetics for the reduction of the cluster, $k_{app} = (9.4 \pm 0.3) \cdot 10^9\ M^{-1} \cdot s^{-1}$. The second phase follows an intramolecular first-order reaction $k_B = (8.3 \pm 1.7) \cdot 10^2\ s^{-1}$ which is observed as a further reduction of the active site. Spectral changes accompanying the reduction of oxidized spinach ferredoxin in the ultraviolet and visible range are discussed.

Introduction

The properties of a particular free radical or its involvement in a series of reactions can sometimes by studied by the use of high-energy ionizing radiation. Conventionally, the radiation can be used to generate low concentrations of free radicals under steady-state conditions, as in ^{60}Co radiolysis, or to create high concentrations of primary radicals in a very brief time, as in pulse radiolysis. The evaluation of the reaction mechanism under steady-state conditions depends upon the determination of yields of the stable end product(s) of the reaction(s). For example, oxidized ferredoxin can be reduced under steady-state conditions with the CO_2^- radical and the resulting spectrum of the reduced form is determined after termination of radiolysis. The pulse radiolysis method, because of its short resolution times (nanoseconds and shorter), makes it possible to study short-lived transients. Extensive research on these techniques over the last two decades has shown that by choosing appropriate primary radical scavengers and well designed experimental conditions, specific free radicals can be generated in relatively high concentrations (practical range 1–50 μM) in the presence of enzymes and proteins without damage to the latter [1,2]. Because most primary radical reactions proceed at rates which are near diffusion controlled, the yields and basic reaction mechanisms observed by the steady-state and pulse methods are, within certain experimental limits, the same.

A number of isolated pulse radiolysis studies have been reported on radical-forming compounds (FAD [3], riboflavin [4], flavodoxin [5], NAD and NADP [6–19], ubiquinone [11–13] and proteins (ferredoxin) [14]) known to occupy key positions in photosynthesis. The present program is an attempt to coordinate and extend this scattered information into a coherent framework which would serve as a foundation for a systematic study of electron-transport reactions under complex situations in the presence of proteins and enzymes.

The first phase of the program will be focused on

the study of electron-transfer reactions in the $NADP^+$-reducing system (ferredoxin → flavoprotein → NADP/NADPH). Although reduction and denaturation of ferredoxin under radiolytic conditions had been studied earlier [14,15], we found it necessary to reinvestigate this protein in greater depth and under our experimental conditions, since our preliminary results indicated significant differences in the rate of reaction with e^-_{aq}.

Spinach ferredoxin (Fd) is an electron-carrier protein found in chloroplasts, the active site of which is generally represented as a dinuclear Fe_2S_2 cluster liganded to four protein cysteinyl sulfurs [16,17]. The redox potential and stoichiometry of electron transfer as well as the coordination structure of the oxidized ($[Fe_2S_2(S\text{-}Cys)_4]^{2-}$) and reduced ($[Fe_2S_2(S\text{-}Cys)_4]^{3-}$) protein have been determined [16–19].

Although the kinetics of electron transfer from reduced ferredoxin to a number of oxidizing agents [20–22] and from reducing agents such as sodium dithionite to oxidized ferredoxin [23] have been investigated, relatively little was known about the reactivity of oxidized ferredoxin toward very strong reduceding agents such as CO_2^- and in particular e^-_{aq}.

In the present investigation, ferredoxin reduction by CO_2^- and e^-_{aq} was studied using steady-state (^{60}Co γ-irradiation) and pulse radiolysis (pulsing 2 MeV Van de Graaf accelerator).

Materials and Methods

Spinach ferredoxin (type III, Sigma Chemical Co.) was purified as described by Petering and Palmer [24]. The purity index (absorbance ratio A_{420}/A_{276nm}) of the protein was greater than 0.47. Before use in radiolysis experiments, Tris-HCl buffer (0.15 M) was removed from protein samples by dialysis at 4°C against 0.01 M phosphate, pH 7.1. Control experiments had shown that formate (up to 1 M), oxalate (up to 50 μM) and *tert*-butanol (up to 1 m) had no deleterious effect upon ferredoxin over several hours.

Sodium formate was prepared by three recrystallizations in the presence of EDTA. *tert*-Butanol was purified by distillation (20 theoretical plate column) followed by two recrystallizations from a melt, and a final distillation. All aqueous solutions were prepared by adding concentrated ferredoxin to 1.0 M aqueous *tert*-butamol for e^-_{aq} reactions, and 0.01–1.0 M sodium formate for CO_2^- reactions. Samples were degassed by gently bubbling either ultra high-purity N_2 (for e^-_{aq} studies) or N_2O (for CO_2^- studies) through the solution.

Continuous radiolysis studies were carried out with ^{60}Co γ-ray sources which had an energy output of 3.3 and 0.55 krad/min, respectively. Pulse radiolysis experiments were carried out with a Van de Graaff accelerator, using 1 μs pulses. The data were analyzed using a PDP-11 computer interfaced to both the Van de Graaf accelerator and the photodetector.

G values by definition represent the number of molecules formed or changed per 100 eV of energy absorbed. G values for ferredoxin reduction were calculated from observed absorbance changes at 420 and 465 nm (molar extinction coefficients are given in Table III and ferrous dosimeter calibrations using $G(Fe^{3+}) = 15.6$.

Results and Discussion

Although formate and *tert*-butanol protect ferredoxin against extensive radiation damage, a small fraction is irreversibly altered. Comparison studies of absorbance changes at 276 and 420 nm suggest that the damage occurs at the active center and that the primary protein structure is essentially left unaffected.

The degree of irreversible decomposition under radiation-induced reducing conditons was ascertained by measurement of the percentage of ferredoxin recovered upon reoxidation with molecular oxygen:

$$\text{Percent recovery} = \frac{(A_3 - A_2)}{(A_1 - A_2)} \times 100 \qquad \text{(I)}$$

where A_1 is the initial absorbance at 420 nm before irradiation, A_2 is the absorbance after irradiation, and A_3 represents the absorbance after the irradiated solution has been reoxidized with molecular oxygen.

A study of the protective action of 0.1 and 1.0 M formate as a function of total dose delivered to a given sample is summarized in Table I. The percent recovery also varies between individual batches of protein by about 4–6%. Since in spectral studies the highest dose used was 4.4 krad, the approximate cumulative error is of the order of 8%. In the kinetic studies by pulse radiolysis the irreversible change in protein was negligible, since the dose per pulse

TABLE I

EXTENT OF REDOX SITE DENATURATION IN SPINACH FERREDOXIN (42.1 μM) UPON IRRADIATION WITH ^{60}Co γ-RAYS (0.6 krad/min) IN N_2O-SATURATED SODIUM FORMATE SOLUTIONS AT pH 7.1

Percent recovery of $Fe_2S_2(S\text{-}Cys)_4$ clusters monitored at 420 nm upon reoxidation by molecular oxygen of radiolytically reduced ferredoxin.

Total dose (krad)	Percent recovery	
	0.1 M HCOONa	1.0 M HCOONa
1.12	97.8	98
1.44	96.3	97
3.19	93.3	97
4.24	89.8	95
7.95	84.7	93

delivered to a sample was 0.1 krad or less.

Measured rate constants for the reduction of oxidized ferredoxin by e^-_{aq} are also dependent upon the total dose delivered to a given sample. Fig. 1 illustrates how the observed rate of reaction of e^-_{aq} with ferredoxin increases upon multiple pulsing of the same sample. The experiment was carried out in an N_2-purged 1 M *tert*-butanol solution containing 9.18 μM ferredoxin (oxidized), 0.01 M phosphate

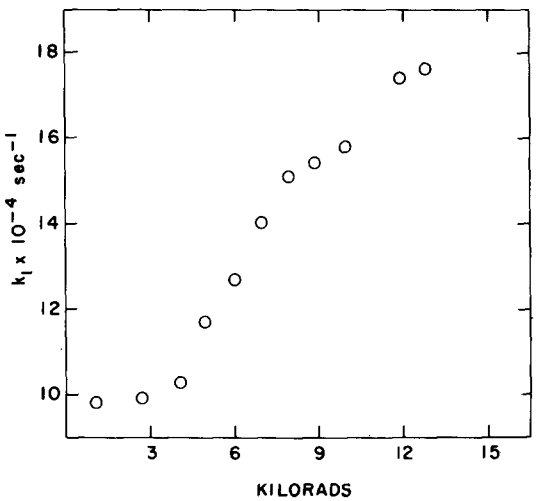

Fig. 1. Reactivity of e^-_{aq} with spinach ferredoxin (9.18 μM) in 1 M *tert*-butanol at pH 7.1 and 24°C. k_A is given here as a function of total radiation dose received by the protein upon repetitive pulsing with 2 MeV electrons.

(pH 7.1) at 24°C. The rate of disappearance of e^-_{aq} was monitored at 700 nm. As is apparent from the initial plateau region in Fig. 1, the observed rate constant stays constant up to a total dose of 3.55 krad beyond which it increases with increasing dose indicative of protein denaturation [25]. In view of these results, all kinetic data were therefore obtained from first pulse reactions only (using fresh samples for each run to insure that the oxidized ferredoxin was in a native state) with energy inputs of less than 0.1 krad/pulse.

As a preliminary to the spectral and kinetic studies by radiation methods, the validity of the Beer-Lambert law was tested for our protein sample at 465, 420, 330 and 276 nm for oxidized ferredoxin and at 465 and 420 for reduced ferredoxin obtained by dithionite reduction. The absorbance was found to be a linear function of ferredoxin concentration over the ranges measured; 1.6–37 μM for oxidized ferredoxin and 3.0–31 μM for reduced ferredoxin.

Reduction of oxidized ferredoxin with CO_2^- radical

The CO_2^- radical is formed when an aqueous formate solution saturated with N_2O is exposed to ionizing radiation (numbers in parentheses are G values for pure water):

$$H_2O \rightsquigarrow OH(2.74), e^-_{aq}(2.76), H(0.55), H_3O^+(2.76), H_2(0.45), H_2O_2(0.72) \quad (II)$$

$$N_2O + e^-_{aq} + H_2O \rightarrow N_2 + OH^- + OH \quad (1)$$

$$HCOO^- + OH \rightarrow CO_2^- + H_2O \quad (2)$$

$$HCOO^- + H \rightarrow CO_2^- + H_2 \quad (3)$$

In the absence of reacting substances CO_2^- dimerizes above pH 5 to oxalate at a rate $k_4 = 10^8\ M^{-1} \cdot s^{-1}$ [25]:

$$CO_2^- + CO_2^- \rightarrow \begin{matrix} COO^- \\ | \\ COO^- \end{matrix} \quad (4)$$

With ferredoxin (F_d) the CO_2^- radical reacts at a rate $k_5 = (6.2 \pm 0.6) \cdot 10^7\ M^{-1} \cdot s^{-1}$.

$$CO_2^- + Fd_{ox} \rightarrow CO_2 + Fd_{red} \quad (5)$$

As is apparent from the rate constants for reactions 4 and 5, relatively high concentrations of ferredoxin would have to be used under pulse radiolytic condi-

TABLE II

CHEMICAL YIELDS OF FERREDOXIN REDUCTION BY CO_2^- AS A FUNCTION OF pH IN AN N_2O-SATURATED 1 M FORMATE SOLUTION CONTAINING 40 μM OXIDIZED FERREDOXIN

pH	G (Fd_{red})
5.05	5.88 ± 0.05
7.10	5.85 ± 0.05
9.67	5.81 ± 0.05

tions to compete effectively with reaction 4 and reduce oxidized ferredoxin quantitatively.

Stoichiometric reduction of oxidized ferredoxin by CO_2^- is favorable under conditions of a low steady-state concentration of CO_2^-, as is the case in ^{60}Co γ-radiolysis. Chemical yields, $G(Fd_{red})$, determined as a function of energy input in 1 M formate at different pH values are listed in Table II. In these experiments, N_2-purged samples were irradiated in sealed 1 cm suprasil cells by ^{60}Co γ-rays (0.55 krad/min). The reduction of ferredoxin was monitored at 420 nm. As is apparent, within experimental error the pH does not affect the chemical yield over the range studied. The average yield of $G(Fd_{red}) = 5.85$

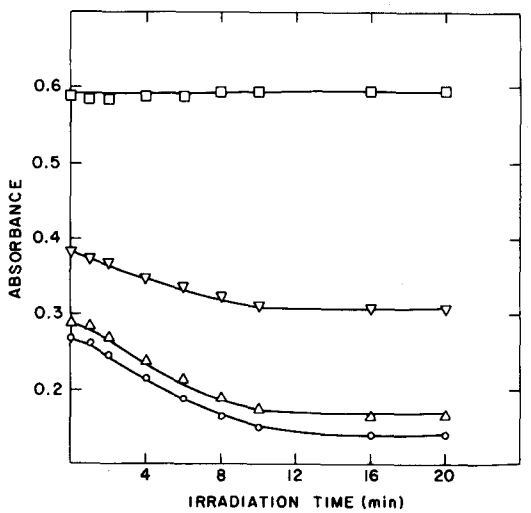

Fig. 2. Absorbance of spinach ferredoxin at various wavelengths as a function of irradiation time (about 4 μM CO_2^-/min). The solution contained 36.8 μM ferredoxin in 1.0 M formate saturated with N_2O at pH 7.3. Wavelengths: □, 276 nm; △, 330 nm; △, 420 nm; ○, 465 nm.

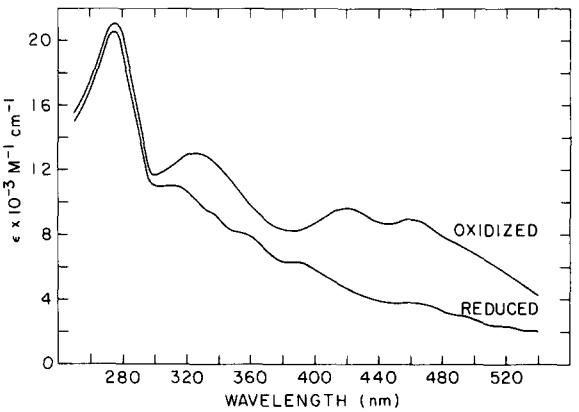

Fig. 3. Absorption spectra of oxidized and radiolytically reduced forms of spinach ferredoxin. 1.0 M formate saturated with N_2O at pH 7.1 and 24°C. The original concentration of oxidized ferredoxin was 17.8 μM.

is low by about 10–15% if one takes into consideration that $G(CO_2^-)$ increases with increasing formate concentration. In N_2O-saturated solutions $G(CO_2^-)$ varies from 6.0 in 1 mM formate to 7.3 in 1 M formate solution due to track scavenging [26,27]. The lowering of $G(Fd_{red})$ can be partially accounted for by the observation that in this system H_2O_2 (molecular yield) is destroyed by CO_2^- in a ratio of $2H_2O_2/CO_2^-$.

To verify the $G(Fd_{red})$ value, the spectrum of reduced ferredoxin formed in reaction 5 and the corresponding molar absorbances were obtained by the following alternative method. A solution of known oxidized ferredoxin concentration, saturated with N_2O and containing 1 M formate at pH 7.1, was irradiated for increasing times in a ^{60}Co γ-ray

TABLE III

MOLAR EXTINCTION COEFFICIENTS OF RADIOLYTICALLY REDUCED SPINACH FERREDOXIN IN 1.0 M SODIUM FORMATE AT pH 7.3 AND 24°C

Wavelength (nm)	ϵ ($M^{-1} \cdot cm^{-1}$)	ϵ ($M^{-1} \cdot cm^{-1}$) [a]
465	3 680 ± 240	3 700
420	4 560 ± 250	4 550
330	9 820 ± 300	–
276	20 660 ± 540	–

[a] Values taken from Ref. 3.

TABLE IV

SECOND-ORDER RATE CONSTANTS FOR REDUCTION OF OXIDIZED FERREDOXIN WITH CO_2^- AT VARIOUS FERREDOXIN CONCENTRATIONS IN N_2O-SATURATED 1.0 M FORMATE AT pH 7.3 AND 24°C

$[Fd_{ox}]$ (μM)	k_5 ($M^{-1} \cdot s^{-1}$)($\times 10^{-7}$)
36.8	5.9 ± 0.4
22.0	5.6 ± 0.4
11.0	6.9 ± 0.8
5.5	6.2 ± 0.9

TABLE V

SECOND-ORDER RATE CONSTANTS FOR REDUCTION OF OXIDIZED FERREDOXIN BY CO_2^- MEASURED AT VARIOUS WAVELENGTHS IN N_2O-SATURATED 1.0 M FORMATE CONTAINING 22 μM OXIDIZED FERREDOXIN AT pH 7.3 AND 24°C

Wavelength (nm)	k_5 ($M^{-1} \cdot s^{-1}$)($\times 10^{-7}$)
465	5.7 ± 0.5
420	5.6 ± 0.6
330	6.1 ± 0.6

source which generated about 4 μm CO_2^-/min in the sample. Absorbance changes were monitored at 465, 420, 330 and 276 nm after each irradiation period until no further absorbance changes could be observed indicating completion of ferredoxin reduction (see Fig. 2). Following the total spectrum of reduced ferredoxin was performed on a Cary 14 apparatus as shown in Fig. 3. Under these conditions very little (less than 5%, see Table I) ferredoxin is irreversibly destroyed during the first 10 min of irradiation and assuming that all oxidized ferredoxin had been reduced, molar extinction coefficients were calculated on the basis of the initial amount of oxidized ferredoxin in the sample. The results are in good agreement with values in the literature [3] and are summarized in Table III. It should be noted that this method has the advantage of studying the spectral properties of ferredoxin in the absence of any absorbance due to reductant. Previous attempts at obtaining the ultraviolet-visible absorbance spectrum and relevant extinction coefficients of reduced ferredoxin have been hampered at shorter wavelengths by absorbance due to the reductant, e.g., sodium dithionite.

Rates of reduction of oxidized ferredoxin by CO_2^- were determined under pseudo first-order conditions by pulse radiolysis. The CO_2^- was generated by a 1 μs pulse in an N_2O-saturated 1 M formate solution containing various amounts of ferredoxin. The reactions followed strict first-order kinetics over more than four half-lives and the observed pseudo first-order rate was proportional to the ferredoxin concentration. The corresponding computed second-order rate constants are listed in Table IV. The average value $k_5 = (6.2 \pm 0.6) \cdot 10^7$ $M^{-1} \cdot s^{-1}$ is in fair agreement with a value reported in previous work [15].

Essentially identical rates were observed when the ferredoxin reduction was measured at three different wavelengths (See Table V), suggesting that all spectral changes are due to a single chromophore. Following the initial change in absorbance, no further changes were observed up to 10 s after the pulse.

The reduction of oxidized ferredoxin with e_{aq}^-

The reduction of oxidized ferredoxin with e_{aq}^- was studied by pulse radiolysis (1 μs pulses) in 1 M *tert*-butanol solutions. While *tert*-butanol effectively scavenges the hydroxyl radical, the resulting *tert*-butyl radical is inert toward protein and disappears by an independent dimerization process [28,29]. Control experiments have shown that the alcohol if highly purified has no deleterious effect upon ferredoxin over several hours. All rate measurements were carried out under pseudo first-order conditions ($[Fd_{ox}] >> [e_{aq}^-]$) and corrected for the spontaneous decay of e_{aq}^- ($t_{1/2} \approx 34$ μs) in the absence of ferredoxin.

The reduction of ferredoxin by e_{aq}^- is complex, since e_{aq}^- appears to react simultaneously with the active site, $[Fe_2S_2(S\text{-}Cys)_4]^{2-}$, and unknown functional groups of the protein. Experimental observations show that ultimately all e_{aq}^- generated in a given sample leads to stochiometric reduction of oxidized ferredoxin to reduced ferredoxin. The complex behavior of the system is best seen when the overall reduction is monitored at 420 nm where both ferredoxin and e_{aq}^- absorb ($\Delta\epsilon_{Fd_{ox}-Fd_{red}} = 5120\,M^{-1} \cdot cm^{-1}$; $\epsilon_{e_{aq}^-} = 2300\,M^{-1} \cdot cm^{-1}$) [29]. Composite results of measurements taken on different time scales show (Fig. 4) two consecutive reactions labeled A and B.

Absorbance change A, which represents the overall

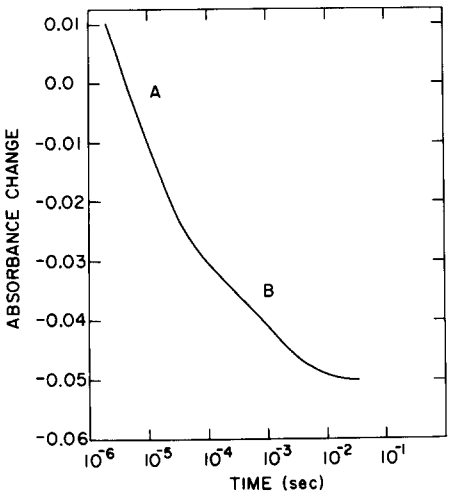

Fig. 4. Observed change in spinach ferredoxin absorbance at 420 nm with time during the reaction of oxidized ferredoxin (9.18 μM) with e^-_{aq} following a 1 μs pulse in 1.0 M *tert*-butanol saturated with N_2 at pH 7.1 and 24°C.

reaction of e^-_{aq} with ferredoxin is more favorably monitored at 700 nm where $\epsilon_{e^-_{aq}} = 18\,000\ M^{-1} \cdot cm^{-1}$ [30] and $\Delta\epsilon_{Fd_{ox}-Fd_{red}} < 300\ M^{-1} \cdot cm^{-1}$. Corresponding pseudo first-order rate constants for the disappearance of e^-_{aq} as a function of ferredoxin concentration are listed in Table VI. The numerical rate values represent averages of four runs per ferredoxin concentration. As is apparent the observed first-order rate constants are proportional to the ferredoxin concentration. Assuming that only one electron (e^-_{aq}) reacts with each ferredoxin molecule, an apparent second-order rate constant can be computed, $k_{app} = (9.4 \pm 0.3) \cdot 10^9\ M^{-1} \cdot s^{-1}$. This value is approx. 3-times smaller than an earlier reported value [15].

A study of the pseudo first-order rate constant for reaction A (7 μM oxidized ferredoxin; 1 M *tert*-butanol; N_2 saturated at pH 7.1 and 24°C) at 700, 465 and 420 nm shows that within experimental error the numerical values are the same: at 700 nm $k_A = (6.4 \pm 0.8) \cdot 10^4\ s^{-1}$; at 465 nm $k_A = (7.0 \pm 0.6) \cdot 10^4\ s^{-1}$ and at 420 nm $k_A = (6.9 \pm 0.8) \cdot 10^4\ s^{-1}$. Also, comparison of the total absorbance change (phase A and B) at 420 and 465 nm indicates that equivalent amounts of proteins are being reduced. For example, the generation of 1.8 μM e^-_{aq} resulted in a $\Delta[Fd_{ox}]$ of 1.7 μM measured at 420 nm and a $\Delta[Fd_{ox}]$ of 1.8 μM at 465 nm.

Inspection of Fig. 4 shows that reactions A and B

TABLE VI

RATE OF REACTION OF e^-_{aq} WITH OXIDIZED FERREDOXIN

Absorbance changes were monitored at 700 nm as a function of ferredoxin concentration in deoxygenated 1 M *tert*-butanol at pH 7.1 and 24°C.

$[Fd_{ox}]$ (μM)	k_A (s^{-1})(X10^{-4})	k_{app} ($M^{-1} \cdot s^{-1}$)(X10^9)
2.74	2.60 ± 0.10	(9.5 ± 0.4)
7.30	6.65 ± 0.15	(9.1 ± 0.2)
15.40	14.90 ± 0.15	(9.7 ± 0.1)

TABLE VII

FIRST-ORDER RATE CONSTANT (k_B) FOR FERREDOXIN BLEACHING AT 420 nm IN DEAERATED 1.0 M *tert*-BUTANOL AT pH 7.1 AND 24°C

$[Fd_{ox}]$ (μM)	$[e^-_{aq}]$ (μM)	k_B (s^{-1})(X10^{-2})
6.0	0.56	10.1 ± 0.3
6.0	1.01	6.8 ± 0.3
6.0	1.69	6.5 ± 0.7
12.0	3.23	10.4 ± 0.8
12.0	15.20	9.3 ± 0.8
18.0	5.80	7.6 ± 0.4
18.0	5.80	7.2 ± 0.4

TABLE VIII

CHANGE IN FERREDOXIN CONCENTRATION AT COMPLETION OF THE INITIAL BIMOLECULAR REDUCTION PROCESS (REACTION 6) AS A FUNCTION OF AMOUNT OF e^-_{aq} ADDED TO SAMPLE

Deaerated 1.0 M *tert*-butanol contained 6.6 μM oxidized ferredoxin at pH 7.1, 24°C. Absorbance changes were monitored at 420 nm.

$[e^-_{aq}]$ (μM)	$\Delta[Fd_{ox}]$ (μM)	$\Delta[Fd_{ox}]/[e^-_{aq}]$
0.56	0.24	0.43
1.01	0.40	0.40
1.69	0.56	0.33

are easily resolved into individual components, since the rates differ by approx. 2 orders of magnitude (cf. values in Tables VI and VII). It can be shown in terms of absorbance changes at 420 nm that at the end of reaction A, approx. 60 ± 7% (average of five runs) of the active site had been reduced. As shown in Table VIII, the percentage of initial active site reduction approaches a limit when $[Fd_{ox}]/[e^-_{aq}] > 6.0$.

The second absorbance decrease (B) in Fig. 4 is due to a first-order process which is 2 orders of magnitude slower than the initial pseudo first-order reaction (A). As can be seen from the results listed in Table VII, this reaction is independent of both $[Fd_{ox}]$ and $[e^-_{aq}]$, and follows first-order kinetics over many half-lives, suggesting that the process is intramolecular and proceeds at an average rate of $k_B = (8.3 \pm 1.7) \cdot 10^2\ s^{-1}$. The possibility that the latter reaction is due to the decay of a radiolytically formed species on the protein surface, such as $RSSR^-$ [30,31], a peptide carbonyl radical anion [32] or an imidazole-type radical [33], was examined by comparing the spectrum of the protein at the end of phase A with the spectrum of the sample after all absorbance change had ceased. Fig. 5 shows that the difference spectrum obtained 200 μs after the electron pulse (e.g., at the end of phase A) is, within experimental error, identical in form to the final spectrum. While absorbance due to $RSSR^-$ can be ruled out, the presence of carbonyl radical anions (at 410 nm) and imidazole radical anions (360 nm) is uncertain because of their weak absorbances.

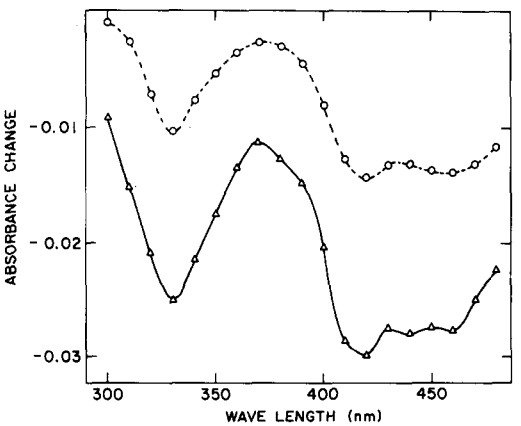

Fig. 5. Change in absorption spectrum of spinach ferredoxin (3.4 μM) due to reaction with e^-_{aq} in 1.0 M *tert*-butanol saturated with N_2 at pH 7.1. o------o, 20 μs after pulse; Δ———Δ, 10 s after pulse.

The absorbance change which occurs subsequent to the disappearance of e^-_{aq} is most likely due to that fraction of electrons which attach themselves to protein molecules (reaction 6) at sites which do not result in immediate cluster reduction [34], and which do not result in a spectral change in the region studied. The negative charge can subsequently tunnel intramolecularly to the redox site, thereby reducing it (reaction 7), as observed in Fig. 4, reaction B:

$$Fd_{ox} + e^-_{aq} \xrightarrow{k_A} \alpha\, Fd_{red} + (1-\alpha)(Fd_{ox} - e^-) \quad (6)$$

$$(1-\alpha)(Fd_{ox} - e^-) \xrightarrow{k_B} (1-\alpha)\, Fd_{red} \quad (7)$$

Such multiple-reaction pathways have been observed in a number of radiolytically reduced proteins [34,35–39]. Since biphasic kinetics are observed only in the case of ferredoxin reduction by e^-_{aq}, the second absorption decrease (phase B, Fig. 4) may alternatively be due to a protein conformational change, induced by the reaction of an electron (but not CO_2^-) [40] with some part of the protein prior to cluster reduction. Conformational changes resulting from radiolytic reduction of proteins have been suggested in the past [41,42]. Recent work has shown that radiolytically reduced (via e^-_{aq}) ferredoxin is more reactive toward oxidants immediately after reduction (100 μs), suggesting an intermediate in a conformationally nonequilibrium state [43].

In conclusion, the redox chemistry of spinach ferredoxin has been found to be amenable to analysis by radiolysis. Reduction by both CO_2^- and e^-_{aq} was found to be essentially quantitative, with minimal protein degradation. Reduction by CO_2^- occurred rapidly ($k_5 = 6.2 \cdot 10^7\ M^{-1} \cdot s^{-1}$) by a single pathway, whereas reduction by e^-_{aq}, in addition to being approx. 100-fold faster, resulted in a biphasic optical decay. No intermediates corresponding to radical anions of amino acids could be detected between 300 and 480 nm, on time scales comparable to that of the second absorbance decrease (Fig. 4B), suggesting either delocalization of charge on the protein, or a conformational change induced by the reaction of an electron with an amino acid residue, prior to redox site reduction.

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