PULSE RADIOLYSIS STUDY OF THE REDUCTION OF SPINACH FERREDOXIN

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1. Introduction

In the reduction pathways of redox proteins by hydrated electrons a clear difference was observed between the oxidases such as laccase [1], ceruloplasmin [2], or xanthine oxidase [3] and that of electron mediating proteins such as the *c* type cytochromes [4, 5] and azurin [6]. Whereas in the latter group of proteins, the redox active center (heme, Cu (II)) is reduced in a direct bimolecular reaction, an indirect multistep mechanism is found to be operative in the oxidases. Ferredoxins are non heme iron electron transfer proteins involved in a wide variety of redox processes in bacteria, plants and higher organisms [7–9]. It is of interest to study the mechanism by which members of this group of electron mediating proteins is reduced by hydrated electrons especially in view of the structural data which are now available for some of these proteins from X-ray crystallography [10–12]. Here, the results of a pulse radiolysis study of the reduction of spinach ferredoxin by hydrated electrons (e^-_{aq}) and the CO_2^- radical ions are reported. These results show that both e^-_{aq} and CO_2^- are reducing the redox center of ferredoxin directly and without any detectable intermediary steps. This observation substantiates the previously observed difference in the reduction mechanism existing between oxidases and electron transfer proteins and is a further illustration of the capacity of the hydrated electron as a probe for the accessibility of redox sites.

2. Materials

Spinach ferredoxin, prepared according to Tagawa and Arnon [13], was supplied by Sigma Chemical Co., St. Louis, Mo. USA and was further purified on DEAE cellulose anion exchange columns (Whatman DE 32, alkali washed and equilibrated with 0.18 M NaCl, 0.15 M Tris-HCl pH 7.3) up to a product having A_{420}/A_{274} = 0.47. All solutions were prepared in triple-distilled water from which O_2 had been removed by prolonged bubbling of highly purified argon or N_2O. The concentration of Tris-HCl in the protein stock solution was reduced to $\simeq$ 0.01 M by ultrafiltration prior to their dilution into the reaction syringes. This was found necessary in view of the observed reactivity of the Tris cation with hydrated electrons. Since 0.1 M tertiary butanol was found to slowly cause denaturation of the ferredoxin, sodium formate had to be used as a scavenger for ·OH radicals.

3. Methods

The experimental details involved in the pulse radiolysis technique have previously been described [14, 15]. Experiments were carried out on the electron accelerator of the Hebrew University of Jerusalem operating at 5 MeV and 200 mA. Different doses were obtained by varying the pulse length from 0.4 to 1.0 μsec. The inductive current caused by the passage of the electron pulse through a coil was used to monitor the pulse intensity. A rectangular (1 × 2 × 4 cm), high purity quartz, flow through cell was used. An optical path of 124 mm was obtained by three passes of the analysing light perpendicular to the incident electron beam. The anaerobic cell-filling method was based on that originally developed by Christiansen et al. [16]. Transmittance changes were recorded by photographing the oscilloscope traces and evaluated by a procedure described previously [4].

4. Results and discussion

In 0.1 M HCOONa (pH 7.5), argon saturated solutions of ferredoxin, the e^-_{aq} absorption band formed with the pulse was found to decay in a pseudo first order process. In fig. 1 the transmittance changes at 550 and 420 nm immediately following the pulse are shown. At the higher wavelength (fig. 1a) the dominant change is due to the appearance and decay of e^-_{aq}. However, since the ferredoxin chromophore is significant also at this wavelength, the transmittance reaches a plateau at a higher level than that of the pre-pulse value. This effect is more pronounced in fig. 1b where the increase in transmittance is scanned at 420 nm. Here, the reduction of the ferredoxin chromophore leads to a more extensive net increase in transmittance. In both these figures the facts that the decay of the e^-_{aq} and the reduction of the ferredoxin proceed in a single, and apparently the same step, is illustrated. The specific rates evaluated for the decay of e^-_{aq} absorption and that due to the ferredoxin chromophore are summarized in table 1. From the close similarity of the specific rates of the observed processes one may conclude that the hydrated electrons reduce the redox site of ferredoxin in a direct reaction. Still, the possibility of very short lived intermediates ($T_{½} < 10^{-7}$ sec) cannot be excluded, owing to the limited resolution of the present equipment. The fraction of ferredoxin reduced via this direct pathway is relatively small and only reaches 25% of the e^-_{aq} produced. Low yields of reduction in the direct step were also observed for two other electron mediating proteins, namely for horse heart cytochrome *c* [4] and *Pseudomonas* azurin [6]. This inefficiency results from reactions of e^-_{aq} with other groups on the protein and may reflect the limited cross section for the direct reduction. The specific rate of reduction of ferredoxin by e^-_{aq} is significantly smaller than those observed for azurin and cytochrome *c* having similar molecular weights. This seems to be the effect of the excess of negative electric charges carried on the ferredoxin molecule [8]. The reported specific rates were measured at a relatively high ionic strength of 0.1 M formate ions. Thus at lower electrolyte concentrations a decrease in the specific rate of ferredoxin reduction should be expected.

Following the direct fast reaction with the e^-_{aq} a second phase of transmittance increase throughout the absorption bands of the ferredoxin chromophore is observed in the time range of a few milliseconds. This reduction step was also found to be a pseudo first-order reaction and the observed specific rate is

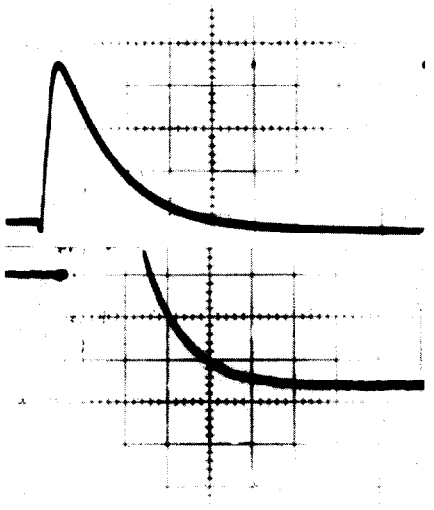

Fig. 1. Oscilloscope traces of the transmittance changes observed in 5.8×10^{-6} M spinach ferredoxin solution (argon saturated and 0.1 M HCOONa), following the electron pulse. Sweep rate in both traces is 5 μsec/scale unit. a) At 550 nm, vertical 50 mV/div. (I_0 = 805 mV). b) At 420 nm, vertical scale 50 mV/div. (I_0 = 850 mV).

Table 1
Specific rates of the decay of e^-_{aq} and of the ferredoxin chromophore.

[Ferredoxin] M $\times 10^6$	[e^-_{aq}] M $\times 10^6$	k (M^{-1} $sec^{-1} \times 10^{10}$) 550 nm	420 nm	400 nm	460 nm
5.8	2.0	2.7 ± 0.3	3.0 ± 0.3	–	–
9.0	2.0	2.9 ± 0.2	3.0 ± 0.3	3.2 ± 0.3	3.0 ± 0.3
9.0	4.8	2.9 ± 0.2	3.1 ± 0.3	–	–

Experiments carried out in argon saturated, 0.1 M HCOONa solutions (pH 7.5). Temperature = 20 ± 2°C.

$(8.0 \pm 0.7) \times 10^7$ M^{-1} sec^{-1}. This is assumed to be the reduction by CO_2^- radical ions. To check on this, the following reaction sequence was used to convert both hydrated electrons and OH radicals into CO_2^- radicals:

$$N_2O + e^-_{aq} \rightarrow N_2O^- \rightarrow N_2 + \cdot O^-(\cdot OH).$$

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

Indeed, in ferredoxin solutions containing 0.1 M HCOONa and saturated with N_2O the fast (several μseconds) reduction phase disappeared completely whereas only the slow (few mseconds) phase remained (fig. 2). The specific rate of this reaction is $(8.0 \pm 0.7) \times 10^7$ M^{-1} sec^{-1}, namely the same as that observed for the slow phase reduction under argon. A comparison with the specific rates at which cytochrome *c* [5] and ceruloplasmin [17] are reacting with the CO_2^- radical (7.9×10^8 and 4×10^9 M^{-1} sec^{-1}, respectively), shows that ferredoxin is reacting significantly slower. Here again the electrostatic charges on ferredoxin might be the cause for the decrease in reactivity.

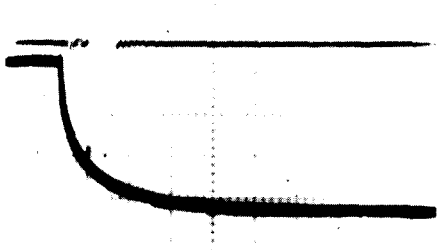

Fig. 2. Oscilloscope trace of the transmittance change at 420 nm in N_2O saturated, 0.1 M HCOONa solution a ferredoxin (6.0×10^{-6} M). Sweep rate is 2 msec/scale div., sensitivity vertical scale 50 mV/cm (I_0 = 820 mV).

In analogy to the proposed direct electron transfer to the heme center of cytochrome *c*, we may assume the existence of a crevice or other form of open channel in the structure of spinach ferredoxin enabling the observed direct reduction of the iron–sulphur cluster. Though no structural data are available for plant ferredoxins, examination of the resolved structures of the related non heme iron electron transfer proteins (Rubredoxin [10], ferredoxin from *Peptococcus aerogenes* [12] and high potential iron protein from *Chromatium vinossum* [11]) supports the assumed accessibility of the redox center to direct reduction.

It is instructive that the non heme iron redox center of xanthine oxidase was found non reacting with e^-_{aq} [3]. This behaviour is well paralleled in the inertness of the type 1 Cu (II) of laccase and ceruloplasmin [1, 2] versus its direct reduction in azurin [6].

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