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Transient kinetic and oxidation-reduction studies of spinach ferredoxin : nitrite oxidoreductase

Masakazu Hirasawa [a], Gordon Tollin [b], Zdzislaw Salamon [b], David B. Knaff [a,c,*]

[a] *Department of Chemistry and Biochemistry, Texas Tech University, Lubbock, TX 79409-1061, USA*
[b] *Department of Biochemistry, University of Arizona, Tucson, AZ 85721, USA*
[c] *Institute for Biotechnology, Texas Tech University, Lubbock, TX 79409-1061, USA*

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Abstract

The oxidation-reduction midpoint potentials for the two prosthetic groups of the chloroplast-located, ferredoxin-dependent nitrite reductase of spinach leaves have been determined by spectroelectrochemical titrations and cyclic voltammetry. The average of the results obtained by the two techniques are $E_m = -290$ mV for the siroheme group and $E_m = -365$ mV for the [4Fe-4S] cluster. The value obtained for the [4Fe-4S] cluster is substantially more positive than values obtained previously in experiments which utilized electron paramagnetic resonance spectroscopy at cryogenic temperatures to monitor the reduction state of the cluster. Laser flash photolysis experiments have been used to monitor electron transfer from reduced ferredoxin to nitrite reductase and have provided the first evidence for electron transfer between the two prosthetic groups of the enzyme. The effect of ionic strength on the observed kinetics has provided support for the proposal that electrostatic interactions between ferredoxin and nitrite reductase play an important role in the reaction mechanism.

Key words: Nitrite reductase; Ferredoxin; Oxidation-reduction titration; Cyclic voltammetry; Laser flash photolysis; Protein-protein complex; (Spinach)

1. Introduction

The enzyme ferredoxin:nitrite oxidoreductase (EC 1.7.7.1, hereafter referred to as nitrite reductase) catalyzes the six-electron reduction of nitrite to ammonia, a key step in the assimilation of nitrogen in plants and algae [1]. Nitrite reductase is a soluble enzyme, located in the chloroplast stroma where its physiological electron donor, ferredoxin, is reduced by the light-dependent reactions of the non-cyclic electron transfer chain [1]. Ferredoxin-dependent nitrite reductases, which have been isolated from a number of higher plants and algae, are monomeric proteins with molecular masses ranging from 60 to 64 kDa and contain a single siroheme and a single [4Fe-4S] cluster as prosthetic groups (Refs. [1,2] and references cited therein). Amino-acid sequences have been deduced from cDNA sequences for the spinach, corn and tobacco enzymes [3–5]. It is known that the interaction between nitrite reductase and ferredoxin is electrostatic in nature, with ferredoxin supplying the negative charges and nitrite reductase the positive charges involved in the interaction between the two proteins [1,6–8]. Oxidation-reduction titrations [2,9–12] and extensive spectroscopic [10,12–21] and ligand binding [8–10,12–23] studies have been carried out on ferredoxin-dependent nitrite reductases. Although some possible intermediates on the pathway from nitrite to ammonia have been proposed [10,13, 14,16], no detailed kinetic studies of the enzyme have yet been performed. Below we present data on the kinetics of electron transfer from the deazariboflavin radical and from reduced spinach ferredoxin to spinach

* Corresponding author; address c. Fax: +1 (806) 7421289.

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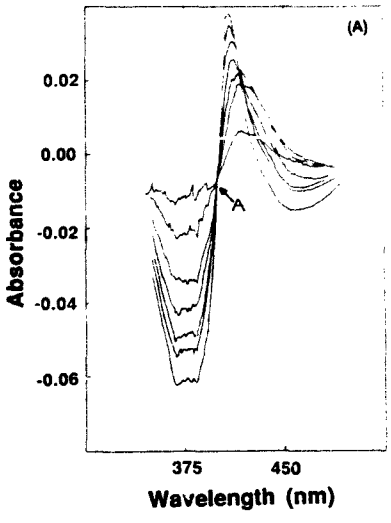

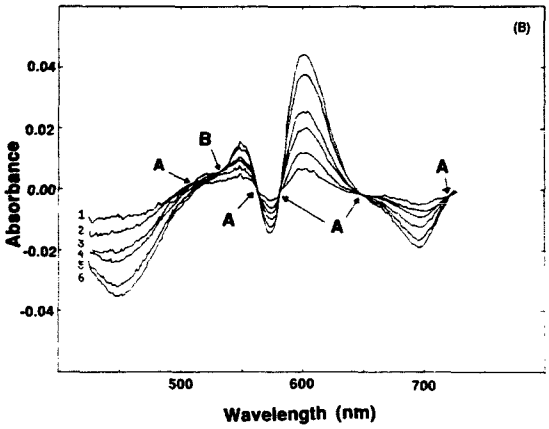

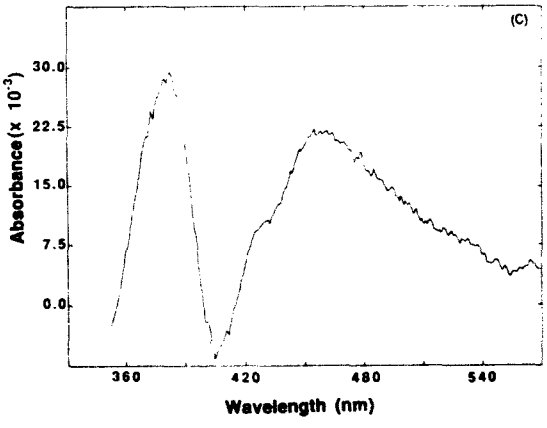

nitrite reductase and provide new evidence on the redox properties of the [4Fe-4S] cluster in the enzyme.

2. Materials and methods

Ferredoxin and nitrite reductase were purified from spinach leaves and stored as described previously [7]. The cross-linked complex between nitrite reductase and ferredoxin was prepared as described previously [7]. The purity of the proteins was ≥ 95%, using criteria described previously [7], and the specific activities of the nitrite reductase preparation and of the cross-linked ferredoxin/nitrite reductase complex used for these experiments, assayed as described previously, were essentially identical to those reported previously [7]. Ferredoxin concentrations were estimated from the absorbance at 422 nm using an extinction coefficient of 9.7 mM^{-1} cm^{-1} [24] and nitrite reductase concentrations were measured as described previously [7].

Absorbance spectra were measured using a Shimadzu Model UV2100U spectrophotometer or an OLIS modification of a Cary 15 spectrophotometer. Laser flash photolysis measurements were carried out with anaerobic samples, using the flash photolysis apparatus and methodology described previously [25,26]. Briefly, photolysis of a solution of 5-deazariboflavin and EDTA produces the deazariboflavin semiquinone, which reduces oxidized protein in competition with its own disproportionation. All kinetic experiments were performed under pseudo-first-order conditions, with protein being present in large excess over the photogenerated deazariboflavin semiquinone.

Spectroelectrochemical oxidation-reduction titrations of nitrite reductase were conducted in the cell compartment of a Shimadzu UV2100U spectrophotometer using a gold mini-grid optically transparent electrode and potentiostat system, as described previously [11]. Self-assembled lipid bilayer membranes were deposited on a gold electrode from a membrane-forming solution containing 2.5 mg/ml egg lecithin [PC, phosphatidylcholine, Sigma Chemical Co.) in squalene

Fig. 1. Reduced *minus* oxidized difference absorbance spectra of nitrite reductase photoreduced in the presence of deazariboflavin plus EDTA. (A) The anaerobic reaction mixture contained 100 μM 5-deazariboflavin, 1 mM EDTA and 7 μM nitrite reductase in 4 mM potassium phosphate buffer (pH 7.7) in a total volume of 2 ml. (B) Conditions as in (A) except that 14 μM enzyme was present. Photoreduction was accomplished by illuminating the reaction mixtures in a 1 cm pathlength silica cuvette placed 20 cm from a 200 W lamp for 5, 15, 35, 45, 95 and 125 s (A) or 55, 75, 105, 135, 175, and 215 s (B) of total irradiation. The spectra of fully oxidized nitrite reductase and deazariboflavin have been subtracted from each of the spectra shown. (C) The spectrum obtained after 135 s of illumination was subtracted from that obtained after 215 s of illumination.

(Fluka Chemie)/butanol (1:3 v/v) as previously described [27]. A gold wire of 0.5 mm diameter (Aldrich Chemical Co.) with a Teflon sleeve of 0.52 mm inner diameter was used as a working electrode. The reference electrode was a saturated Ag/AgCl electrode. Electrolyte solutions were degassed with high purity argon before use. Cyclic voltammetric experiments were performed as described previously, using a custom-made functional generator with a current interface [28]. Potential and current output were digitized by a Heath-Zenith SD 4850 digital oscilloscope and the waveforms were transferred to the hard disk of an IBM XT computer. A BASIC program performed all data analysis. The applied voltage was scanned over the range from −0.9 to 0.1 V at a rate of 0.020 V/s. The formal redox potentials ($E_{1/2}$) were determined from the average of the oxidative (anodic E_{ox}) and reductive (cathodic E_{red}) peak potentials, i.e., $(E_{ox}+E_{red})/2$. The conversion of the applied potentials to normal hydrogen electrode values were made assuming the standard literature value for the potential of the reference electrode (0.197 V).

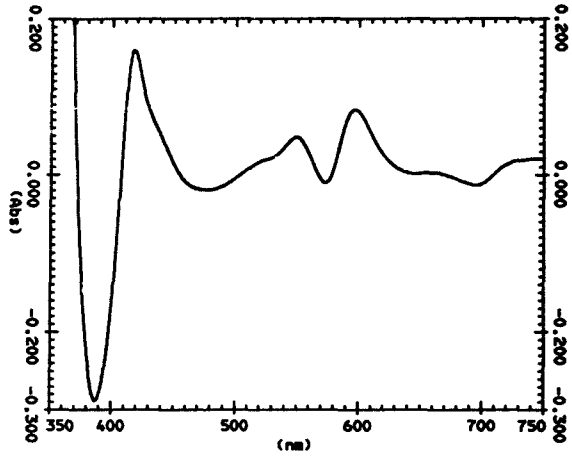

Fig. 2. Reduced *minus* oxidized difference spectrum of chemically reduced nitrite reductase. The sample and reference cuvettes both contained 14.6 μM enzyme in 250 mM potassium phosphate buffer (pH 7.7). The spectrum was recorded after the enzyme in the sample cuvette was reduced by adding a few crystals of sodium dithionite.

3. Results

In most earlier investigations of nitrite reductase in which the siroheme and [4Fe-4S] cluster prosthetic groups were reduced for spectroscopic characterization, sodium dithionite was used as the reductant [10,14,15,17,19,23]. The use of the dithionite anion as a reductant can result in the production of sulfite, which can serve as a ligand to the siroheme [29–33]. We have confirmed earlier observations [29,30] that sulfite can serve both as a siroheme ligand and as a substrate for spinach nitrite reductase (Aketagawa, J. and Hirasawa, M., unpublished observations). Thus, previous studies, in which attempts were made to measure the E_m values of the prosthetic groups of nitrite reductase using dithionite to reduce the enzyme, were likely to have measured E_m values for the sulfite adduct rather than those of the free enzyme. To eliminate this difficulty we have reduced spinach nitrite reductase photochemically in the presence of 5-deazariboflavin plus EDTA, electrochemically in the presence of oxidation-reduction mediators, and via cyclic voltammetry, thus avoiding the use of the dithionite anion as a chemical reductant.

Illumination of anaerobic reaction mixtures containing nitrite reductase, EDTA and 5-deazariboflavin for increasing periods of time produced an increasing extent of reduction of the enzyme. Reduced *minus* oxidized difference spectra are shown in Fig. 1A for the wavelength region below 500 nm and in Fig. 1B for the wavelength region from 425 to 730 nm. The absorbance spectrum of the fully reduced enzyme has peaks at 412, 549 and 604 nm and troughs centered at 387, 449, 572 and 696 nm. The reduced *minus* oxidized difference spectrum of photoreduced nitrite reductase clearly differs from that of the enzyme reduced by dithionite (Fig. 2). These differences may arise from incomplete reduction of the enzyme by dithionite [9,10,12,15,18], from sulfite binding by the enzyme when dithionite is used as the reductant, or from both factors.

Analysis of the time course for the photoreduction reveals that it proceeds in two kinetic phases. The first phase, which is complete under the experimental conditions of Fig. 1 after approximately 105 s, is characterized by isosbestic points located at 400, 497, 563, 581, 640 and 725 nm (marked A in Fig. 1). The second phase, which is complete after approximately 215 s, is characterized by a shift in one of the isosbestic points from 497 to 532 nm (marked B in Fig. 1). This pattern can be interpreted in terms of the presence of two electron-carrying centers in spinach nitrite reductase that are reduced sequentially and that differ by less than 100 mV in their E_m values. This result was surprising in view of previous reports that the E_m values for the [4Fe-4S] cluster of plant, ferredoxin-coupled nitrite reductases are approximately 400 mV more negative than those of the siroheme group of the enzymes [9,10,12]. Our results are, however, quite similar to those obtained for the heme subunit of *E. coli* sulfite reductase, a protein with prosthetic group content identical to that of spinach nitrite reductase and for which the E_m values of the siroheme and [4Fe-4S] cluster differ by only 70 mV [31]. Although there is some overlap between the two phases, the spectrum of the first photoreduction phase suggests that it is associ-

ated with the reduction of siroheme, as would be expected if the siroheme has a more positive E_m value than that of the [4Fe-4S] cluster. The spectrum of the second phase (Fig. 1C) in the region above 430 nm is consistent with reduction of the [4Fe-4S] cluster in nitrite reductase.

Our unexpected observation that the photoreduction pattern was consistent with the presence of two prosthetic groups in nitrite reductase that do not differ greatly in E_m values, suggested that it would be important to redetermine the E_m values for siroheme and the [4Fe-4S] cluster in oxidation-reduction titrations, taking care to ensure that the E_m values for both centers were measured under identical conditions. Fig. 3 shows the results of a spectroelectrochemical oxidation-reduction titration of spinach nitrite reductase, using $\Delta A_{430\ nm-457\ nm}$ to monitor the oxidation state of the [4Fe-4S] cluster. This wavelength pair was chosen to maximize absorbance changes due to reduction of the cluster, minimize absorbance changes arising from siroheme reduction and eliminate contributions to the difference spectrum arising from reduction of the oxidation-reduction mediators used in the titration. An E_m value of -360 ± 15 mV ($n = 1.2 \pm 0.2$) was obtained for the component being titrated and the difference spectrum observed (not shown), which was similar in the region above 430 nm to that obtained for the second phase during the photoreduction of the enzyme, is consistent with that expected for reduction of a [4Fe-4S] cluster. Oxidation-reduction titrations of the siroheme group in nitrite reductase under conditions identical to those used for the experiment of Fig. 3 (not shown), confirmed our earlier observation [11] that the E_m value of the siroheme group of the enzyme is -305 mV.

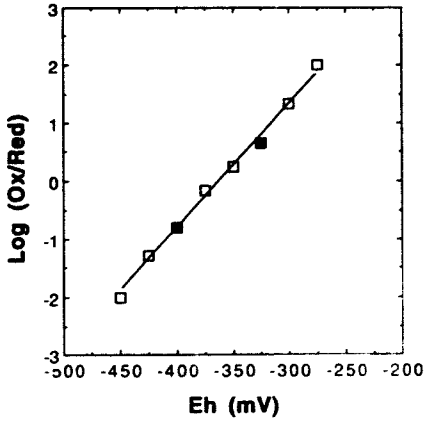

Fig. 3. Oxidation-reduction titration of the [4Fe-4S] cluster of nitrite reductase. The reaction mixture contained 100 μM nitrite reductase and 80 mM NaCl in 10 mM potassium phosphate buffer (pH 7.7) and the following oxidation-reduction mediators: 20 μM anthraquinone-1,5-disulfonate; 20 μM anthraquinone 2-sulfonate; 5 μM safranin O; 20 μM benzyl viologen; 20 μM methyl viologen;and 20 μM triquat. The ambient potential was adjusted using the spectroelectrochemical apparatus described in Materials and Methods. Absorbance spectra were measured over the potential range from -200 mV to -500 mV and stored in the memory of the Shimadzu UV2100U spectrophotometer. The spectrum obtained for a sample poised at an ambient potential of -200 mV was taken as the baseline and subtracted from all spectra obtained at lower ambient potentials. The oxidation state of the protein was monitored by following difference absorbance between 430 and 457 nm. Points were taken in both the oxidative (closed squares) and reductive (open squares) directions. Optical pathlength, 0.33 mm; temperature 4°C. The data were plotted according to the Nernst Equation, assuming that the absorbance spectrum measured for a sample poised at $E_h = -275$ mV represented that of nitrite reductase containing a fully oxidized [4Fe-4S] cluster.

Similar E_m values to those obtained by spectroelectrochemical titrations, showing the considerably smaller difference in E_m values between the prosthetic groups than had been reported previously, were obtained from the cyclic voltammetry experiments. The cyclic voltammogram of nitrite reductase at a self-assembled PC bilayer on a gold electrode shows two electrochemical waves (Fig. 4). The half-wave values calculated from the results shown in Fig. 4 are -270 ± 30 mV and -370 ± 30 mV, respectively. Although two waves can clearly be seen in Fig. 4, the fact that they are separated by only ca. 100 mV results in overlap, and makes calculation of the half-wave potentials somewhat less accurate than those obtained from the spectroelectrochemical titrations. If we assume that the more electropositive component seen in the cyclic voltammogram corresponds to the reduction of siroheme, then the E_m values obtained by spectroelectrochemical titrations and cyclic voltammetry are identical within the experimental uncertainties. The discrepancy between the E_m values of -360 mV and -370 mV obtained for the [4Fe-4S] cluster of spinach nitrite reductase obtained in this study using spectroelectrochemical techniques and cyclic voltammetry, respectively, and the much more electronegative values found in our own earlier studies [9] and those of others [10,12] may arise from the fact that the titrations reported herein did not involve dithionite as a reductant, avoiding complications arising from the formation of an enzyme/sulfite adduct, and/or from the fact that earlier titrations all involved sample observation at cryogenic temperatures and, in several cases, were performed at alkaline pH values. In contrast, the experiments of Figs. 3–5 were carried out at a pH closer to neutrality and, as they were also carried out at ambient temperature, the possibility of shifts in E_m, E_h or pH which might arise from lowering the sample temperature to 20 K has been eliminated.

It is possible to utilize the deazariboflavin radical, generated rapidly by a laser flash, to examine the kinetics of electron transfer reactions in enzyme systems involving ferredoxin [25,34–36]. Fig. 5 shows the results of an experiment utilizing this technique in the

absence of ferredoxin, in which the oxidation state of nitrite reductase was monitored at 604 nm, a wavelength at which absorbance changes arise primarily from changes in the oxidation state of siroheme (See Figs. 1 and 2). The transients observed are biphasic and Fig. 6A illustrates the difference spectra for the overall change observed at long times (i.e., 50 ms after the flash) and for the fast (2 ms after the flash) and slow (2 ms to 50 ms) phases. These spectra are consistent with an electron transfer sequence in which the fast phase arises from the reduction of the nitrite reductase siroheme by the photoreduced deazariboflavin semiquinone and the slow phase represents an additional siroheme reduction process occurring via internal electron transfer within the enzyme, presumably between the siroheme and the [4Fe-4S] cluster. Thus, consistent with this interpretation, k_{obs} values for the fast phase (measured at 604 nm) were linearly dependent on nitrite reductase concentration (Fig. 6B), giving second-order rate constants of $1.5 \cdot 10^8$ $M^{-1}s^{-1}$

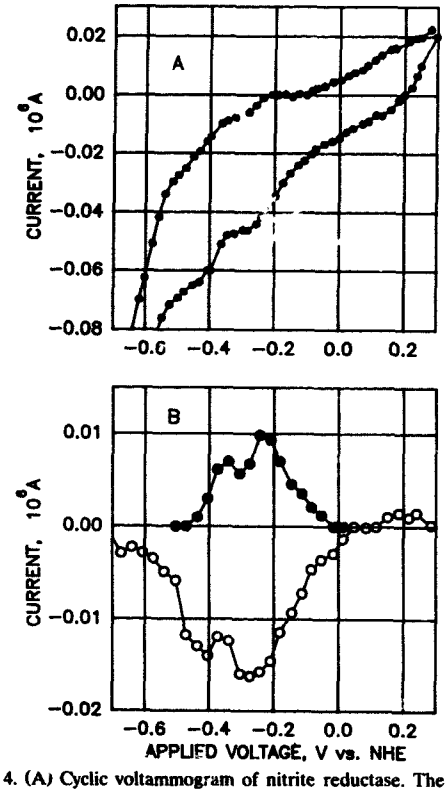

Fig. 4. (A) Cyclic voltammogram of nitrite reductase. The reaction mixture contained 140 μM nitrite reductase in 25 mM potassium phosphate buffer (pH 7.7) containing 25 mM $NaClO_4$. The data were obtained at a scan rate of 20 mV/s with a gold electrode modified with phosphatidylcholine bilayer using 2.5 mg/ml membrane forming solution. (B) Current-voltage reduction (○) and oxidation (●) curves obtained from the cyclic voltammogram of Fig. 4 after background subtraction. All conditions as described in the legend to Fig. 4.

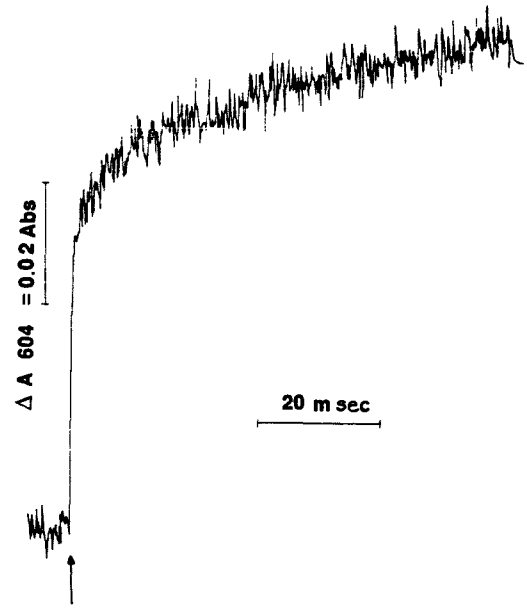

Fig. 5. Kinetic transient for the reduction of nitrite reductase by the deazariboflavin radical. The anaerobic reaction mixture contained 13.9 μM nitrite reductase in 4 mM potassium phosphate (pH 7.7) containing 1 mM EDTA and 100 μM 5-deazariboflavin. The arrow indicates the time of the laser flash.

and $1.0 \cdot 10^8$ M^{-1} s^{-1} at ionic strengths of 39 and 119 mM, respectively. In contrast, the k_{obs} value (100 ± 10 s^{-1}, at an ionic strength of 39 mM) for the slow kinetic phase (measured at 465 nm; cf. Fig 6A) was independent of nitrite reductase concentration (Fig. 6B), as expected for an intramolecular process.

Nitrite reductase is known to form an electrostatically stabilized complex with ferredoxin at low ionic strength, with the complex dissociating at high ionic strength [1,6–8]. The utilization of the photogenerated deazariboflavin radical to study electron transfer between proteins within such complexes has proven to be quite useful [26,34–36] and it thus seemed appropriate to examine the complex between nitrite reductase and ferredoxin by this technique. The reduction of spinach ferredoxin by the photoreduced deazariboflavin radical has been extensively studied [25,34] and kinetic measurements conducted with ferredoxin alone during the course of this investigation (not shown) produced results essentially identical to those previously reported. It was possible, in the presence of nitrite reductase, to follow the time course of both ferredoxin reduction by the deazariboflavin radical and the subsequent reoxidation of reduced ferredoxin by monitoring the absorbance at 500 nm, an isosbestic point for nitrite reductase (see Fig. 1B). Fig. 7A shows the results of such an experiment carried out with nitrite reductase and a 6-fold excess of ferredoxin in a low ionic strength

buffer (4 mM potassium phosphate buffer, pH 7.7), conditions under which the deazariboflavin radical generated by the flash would be most likely to reduce ferredoxin rather than nitrite reductase. It can be calculated, from the observation that the K_d for the complex between ferredoxin and nitrite reductase is < 1 μM at this ionic strength [37] and the assumption that the stoichiometry of the complex is 1:1, that

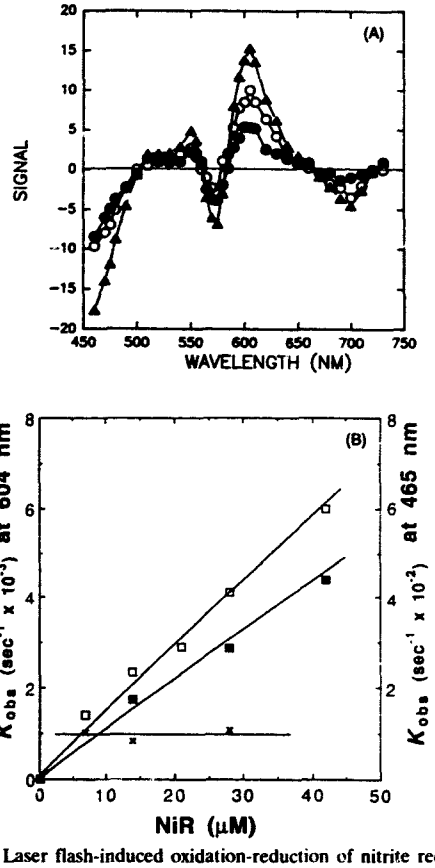

Fig. 6. Laser flash-induced oxidation-reduction of nitrite reductase. (A) Difference spectra. The reaction mixture contained 21 μM nitrite reductase, 1 mM EDTA and 100 μM 5-deazariboflavin in 4 mM potassium phosphate buffer (pH 7.7). Absorbance changes were recorded *vs.* wavelength at 10 nm intervals between 450 and 750 nm, at the indicated times after the flash. (○), (●), and (▲) represent relative absorbance changes (in arbitrary units) for the fast phase (2 ms *minus* zero time), the slow phase (50 ms *minus* 2 ms), and the total absorbance change (50 ms *minus* zero time), respectively. (B) Second-order and first-order plots for the fast and slow phases of nitrite reductase prosthetic group reduction resulting from the laser flash-induced generation of the deazariboflavin radical. Conditions as in (A) except that the nitrite reductase concentration was varied as indicated. Second-order plots for the psuedo-first-order fast phase are shown for data recorded at 604 nm in the presence (closed squares) and absence (open squares) of 80 mM NaCl. A first-order plot is shown for the slow phase with data recorded at 465 nm in the absence of NaCl (×).

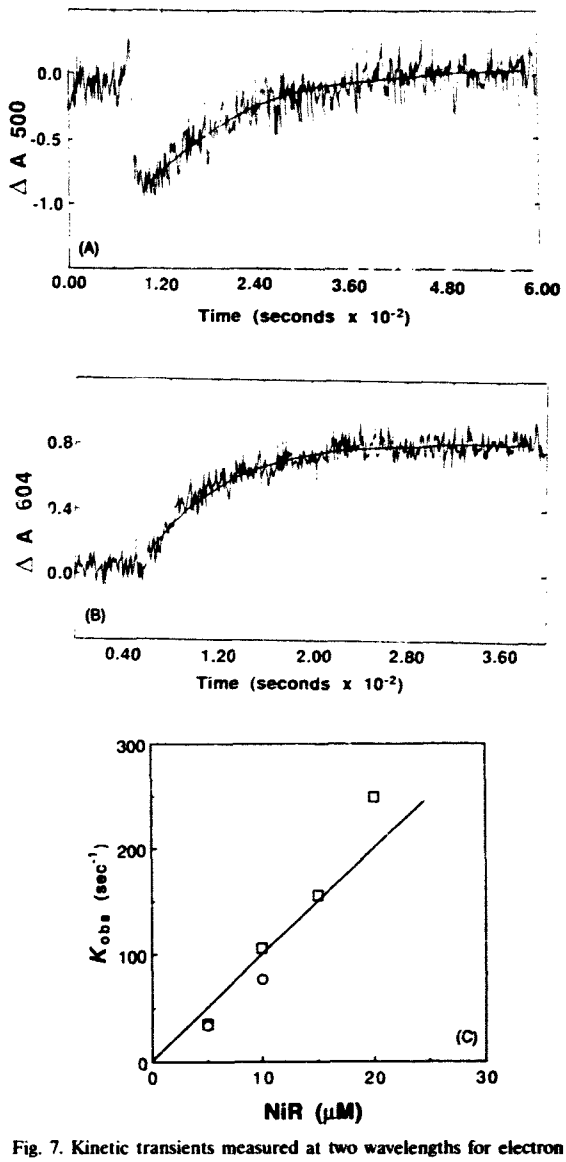

Fig. 7. Kinetic transients measured at two wavelengths for electron transfer between ferredoxin and nitrite reductase. Reaction conditions as in Fig. 6, except that the nitrite reductase concentration was 5 μM and 30 μM ferredoxin was present. (A) 500 nm. (B) 604 nm. Solid lines correspond to single exponential fits to the data. (C) Second-order rate plots are shown for data collected at 604 nm (open circles) and 500 nm (open squares).

essentially all of the nitrite reductase is bound to ferredoxin and virtually no free nitrite reductase is present. As is evident in Fig. 7A, an initial rapid decrease in absorbance at 500 nm was observed (due to ferredoxin reduction), followed by an exponential absorbance increase that returned the absorbance to the pre-flash baseline (due to ferredoxin oxidation). Fig.

7B shows the kinetic transient observed at 604 nm, using the same reaction mixture and reaction conditions. The increase in absorbance at this latter wavelength, which has the same k_{obs} (36 s^{-1}) as that for the absorbance increase at 500 nm (35 s^{-1}), can be attributed to reduction of the siroheme group of nitrite reductase. In this experiment, the siroheme remained reduced for at least 60 ms following the laser flash.

As can be seen from the dependence of k_{obs} on the concentration of nitrite reductase (Fig. 7C), this electron transfer process is second order. Approximately identical second-order rate constants were calculated for the transients at 500 nm, where the absorbance changes are due largely to changes in the redox state of ferredoxin ($1.1 \cdot 10^7$ $M^{-1}s^{-1}$), and 604 nm, where absorbance changes due to changes in the redox state of ferredoxin are small [34] and the absorbance changes are largely due to changes in the redox state of siroheme ($1.0 \cdot 10^7$ $M^{-1}s^{-1}$). If the electron transfer from ferredoxin to the siroheme group of nitrite reductase monitored in these experiments occurs within a tight 1:1 complex between the proteins, one would have expected first-order kinetics, with k_{obs} independent of enzyme concentration, rather than the second-order kinetics actually observed. It is thus likely that the second-order kinetics represent reduction of the siroheme in a pre-formed complex by free reduced ferredoxin generated by the laser flash. Such a reaction has previously been observed for the complex between cytochrome b_5 and cytochrome c [38]. This implies that either the intracomplex electron transfer process is too rapid to be observable in this experiment, or that complexed ferredoxin is inaccessible to reduction by the deazariboflavin semiquinone. Further studies will be required to clarify this point.

Fig. 8 summarizes the ionic strength dependence of the observed rate constant for electron transfer from reduced ferredoxin to the siroheme group of nitrite reductase in a series of experiments in which the ionic strength was varied, either by increasing the concentration of potassium phosphate buffer (upper panel) or by adding NaCl to the basal potassium phosphate buffer (lower panel). The patterns observed when the ionic strength is varied in these two manners differ somewhat quantitatively but are similar qualitatively, with k_{obs} exhibiting a pronounced optimum at intermediate ionic strength. It should also be noted that the V_{max} for the ferredoxin-dependent activity of nitrite reductase, measured during steady-state assays, exhibits considerably less dependence on ionic strength than does k_{obs} (Fig. 8).

It is possible to cross-link ferredoxin to nitrite reductase using a water-soluble carbodiimide [7,8]. We have confirmed our earlier observation [7] that the cross-linked complex between nitrite reductase and ferredoxin is enzymatically active in steady-state assays

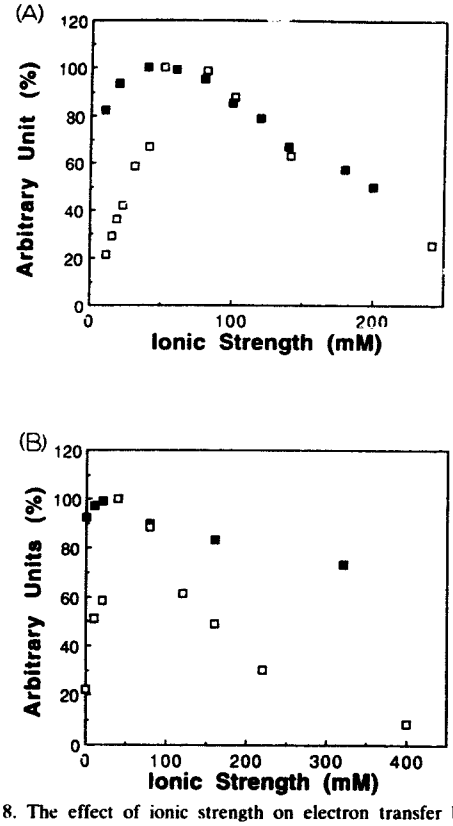

Fig. 8. The effect of ionic strength on electron transfer between ferredoxin and nitrite reductase. Reaction conditions for the laser-induced kinetic transients measured at 604 nm (open squares), were as described in Fig. 7. (A) The ionic strength was adjusted by varying the concentration of the potassium phosphate buffer. (B) The ionic strength was adjusted by adding aliquots of an anaerobic 5 M sodium chloride solution to the reaction mixture. The steady-state data (closed squares) were obtained using the assay system described in Materials and Methods.

in the absence of free ferredoxin, when dithionite is added to reduce the ferredoxin in the covalent complex (data not shown). Fig. 9 shows a typical kinetic transient for the reduction by the photogenerated deazariboflavin radical (Fig. 9A) and subsequent reoxidation of ferredoxin (Fig. 9B) in the cross-linked complex, observed at 500 nm (satisfactory data could not be obtained at 604 nm). Ferredoxin reduction was found to be second order (data not shown), with a rate constant of $1.6 \cdot 10^8$ $M^{-1}s^{-1}$. This can be compared to a value of $6.7 \cdot 10^8$ $M^{-1}s^{-1}$ obtained with the free protein at pH 7.0 and 310 mM ionic strength [34]. Providing that the redox potential of the iron-sulfur cluster of ferredoxin in the cross-linked complex is similar to that of the cluster in the free protein, this would suggest that the accessibility of the [2Fe-2S] cluster to deazariboflavin semiquinone is decreased somewhat within the complex. In contrast, the ob-

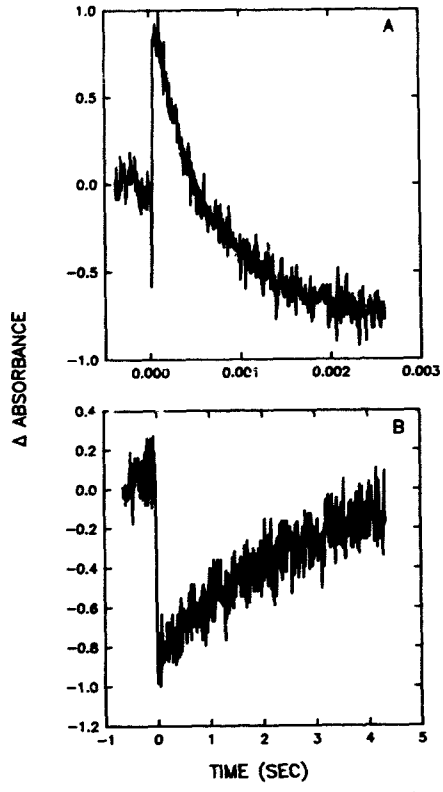

Fig. 9. Kinetic transients for the reduction of the cross-linked ferredoxin/nitrite reductase complex by the deazariboflavin radical. The reaction conditions were as described in Fig. 5, except that the cross-linked ferredoxin/nitrite reductase complex, at a concentration of 28 μM, was present instead of the free enzyme. The data were collected at 500 nm. (A) Fast phase of ferredoxin reduction. (B) Slow phase of ferredoxin reoxidation. Solid lines correspond to single exponential fits to the data.

served rate constant for the reoxidation of ferredoxin was independent of the protein concentration (data not shown), indicating that we are observing an intracomplex electron transfer event. The value for the observed first-order rate constant for intracomplex ferredoxin reoxidation ($0.4 \pm 0.05\ s^{-1}$) under these conditions can be compared with the value that would be obtained in the experiment of Fig. 8 at an equivalent nitrite reductase concentration ($400\ s^{-1}$). This implies that the intracomplex rate constant for electron transfer from ferredoxin to siroheme within the covalent complex is considerably smaller than that for a non-covalent electrostatic or collisional complex. This could be a consequence of the covalent linkage(s) constraining the proteins to assume a less productive orientation. Similar results have been obtained with several other protein-protein complexes [39–43].

4. Discussion

Experiments described above, using photochemical titrations with deazariboflavin, spectroelectrochemical redox titrations and cyclic voltammetry, indicate that the E_m value for [4Fe-4S] cluster of spinach nitrite reductase is considerably more positive than previously reported. The E_m value obtained for [4Fe-4S] cluster in this study from the two different electrochemical techniques, −365 mV, places this prosthetic group of the enzyme approximately midway on the redox scale between ferredoxin, the physiological electron donor to the enzyme ($E_m = -420$ mV, Ref. 1), and the site of reduction of nitrite, the siroheme group of the enzyme $E_m = -290$ mV. As the results obtained from the two different electrochemical techniques employed in this study do not differ by more than the experimental uncertainties involved, it seems appropriate to use the average of the values obtained from spectroelectrochemistry and cyclic voltammetry for both the siroheme and [4Fe-4S] cluster. The difference between the E_m value for the [4Fe-4S] cluster obtained in this study and the values below −500 mV obtained in earlier studies [9,10,12] are likely to arise from the fact that alkaline pH, cryogenic observation temperatures and the use of dithionite as a reductant were avoided in the experiments reported above. If the more positive value obtained in this study is correct, a puzzling problem raised by the earlier results (i.e., that a prosthetic group of the enzyme had an E_m value at least 100 mV more negative than that of the physiological electron donor for the enzyme) has been eliminated.

Previous freeze quench kinetic studies established that the rates of reduction of the siroheme and [4Fe-4S] cluster in spinach nitrite reductase by dithionite are comparable [15]. It should be pointed out that dithionite is a very poor electron donor to the enzyme, supporting nitrite reduction at a rate only 1% of that observed with the physiological electron donor, reduced ferredoxin [15]. The [4Fe-4S] cluster is rapidly reoxidized when nitrite is added to the enzyme, consistent with a role for the cluster in the electron transfer reaction catalyzed by nitrite reductase [15]. We have now been able to demonstrate, using flash photolysis, that internal electron transfer from a group within the enzyme to the siroheme can occur with a rate constant of $100 \pm 10\ s^{-1}$ (see Fig. 6). Inasmuch as the [4Fe-4S] cluster is the only prosthetic group other than siroheme known to be present in the enzyme, it appears very likely that the slow phase of siroheme reduction we have observed when the enzyme is reduced by the deazariboflavin radical reflects electron transfer from this cluster. This observation provides the first evidence for electron transfer between the two prosthetic groups of the enzyme. Although we have not been able to determine the kinetic parameters for the reduction

of the [4Fe-4S] cluster, it appears that cluster reduction by the photoproduced deazariboflavin radical must occur with a rate constant comparable to that for reduction of the siroheme by this radical.

An attempt has been made in these studies to characterize the kinetics of electron transfer from reduced ferredoxin to nitrite reductase. A second-order process, probably corresponding to electron transfer from reduced free ferredoxin to the siroheme of nitrite reductase within a pre-formed ferredoxin/nitrite reductase complex, was observed. Although we have not been able to detect the expected first-order electron transfer from ferredoxin to the enzyme within the complex, one would surmise that this rate constant is larger than the largest observed pseudo-first-order rate constant for the ternary interaction ($k > 250\ s^{-1}$; cf. Fig. 7c). This work has provided new evidence for the importance of electrostatic factors in the interaction between ferredoxin and nitrite reductase, first proposed on the basis of ionic strength effects observed in the course of equilibrium binding studies [1,6–8,37]. The observation that k_{obs} for the reduction of nitrite reductase by reduced ferredoxin decreases markedly as the ionic strength is increased above about 100 mM (Fig. 9) is consistent with the previous hypothesis [6,7,37] that negatively charged groups on ferredoxin interact with positively charged groups on the enzyme during the docking of the two proteins [1]. The sharp decrease in k_{obs} as the ionic strength is decreased below ca. 100 mM probably reflects the transition between a reaction involving free ferredoxin and free nitrite reductase and one involving free ferredoxin and nitrite reductase complexed to ferredoxin. A similar effect was observed in the cytochrome b_5/cytochrome c system [38].

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