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The oxidation-reduction properties of spinach thioredoxins *f* and *m* and of ferredoxin:thioredoxin reductase

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Abstract

Oxidation-reduction midpoint potentials have been determined, using cyclic voltammetry, for the active-site disulfide/dithiol couples of spinach thioredoxins *f* and *m* and of spinach ferredoxin:thioredoxin reductase (FTR) and for a component likely to be the [4Fe-4S] cluster of FTR. Values for the midpoint potentials ($n = 2$) of -210 ± 10 mV were determined for both thioredoxins *f* and *m*. Two redox centers were detected in FTR, with midpoint potential values of -230 ± 10 mV ($n = 2$) and $+340 \pm 30$ mV, respectively. Alkylation of the active-site cysteines of FTR by treatment of the enzyme with *N*-ethylmaleimide (NEM) eliminates the component with the -230 mV midpoint potential, allowing one to assign this value to the active site disulfide/dithiol couple. Inasmuch as the only other electron-carrying center known to be present in FTR is the [4Fe-4S] cluster, it appears likely that the high-potential component can be attributed to this redox moiety. The midpoint potential value of the high-potential feature shifts slightly, to $+380 \pm 20$ mV, in the NEM-treated enzyme.

Keywords: Thioredoxin structure; Ferredoxin:thioredoxin reductase; Active-site disulfide

1. Introduction

The ferredoxin/thioredoxin system plays an important role in regulating carbon metabolism in oxygenic photosynthetic organisms [1,2]. The chloroplasts of higher plants and algae contain two distinct thioredoxins, thioredoxin *f* and thioredoxin *m*. Both thioredoxins are soluble proteins of low molecular mass (about 12 kDa), located in the chloroplast stroma. Thioredoxin *f* selectively activates several enzymes involved in carbohydrate biosynthesis (e.g., fructose-1,6-bisphosphatase, sedoheptulose-1,7-bisphosphatase, phosphoribulose kinase and the $NADP^+$-linked glyceraldehyde-3-phosphate dehydrogenase), while thioredoxin *m* selectively regulates glucose-6-phosphate dehydrogenase and the $NADP^+$-linked malate dehydrogenase [1].

Both thioredoxins *f* and *m* are reduced by the enzyme ferredoxin:thioredoxin reductase (FTR), a heterodimeric $M_r = 25.6$ kDa, soluble protein also located in the chloroplast stroma, via reactions in which two cysteine thiols at the active site of reduced FTR become oxidized to a cystine disulfide while a disulfide on the oxidized thioredoxin is reduced to two cysteine thiols [1]. The reduced thioredoxins in turn reduce regulatory disulfides on their target enzymes to the dithiol state [1]. The source of electrons for the reduction of FTR is reduced ferredoxin [1–3], which is reduced in turn by the light-driven reactions of the Photosystem I reaction center [2]. The interaction between ferredoxin and FTR appears to involve a high-affinity, electrostatically stabilized complex between the two proteins [2–4].

Recently, progress has been made in determining the primary structure of several chloroplast and cyanobacterial thioredoxins [3] and FTR's [5–7]. No three-dimensional

Abbreviations: EPR, electron paramagnetic resonance; FTR, ferredoxin:thioredoxin reductase; NEM, *N*-ethylmaleimide; PC, phosphatidylcholine.

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structural information for any FTR is available. However, the secondary structure of a chloroplast thioredoxin *m* isolated from the green alga *Chlamydomonas reinhardtii* has been determined by ^{1}H-NMR spectroscopy [8] and high-resolution three-dimensional structures are available for both the oxidized [9] and reduced [10] forms of the homologous *Escherichia coli* thioredoxin.

Oxidation-reduction midpoint potential (E_m) values in the range from −230 mV to −260 mV have been measured for the disulfide/dithiol couples of thioredoxins isolated from *E. coli*, *Saccharomyces cerevisiae*, and the bacteriophage T4 [11–14] and an E_m value of −300 mV has been reported for maize thioredoxin *m* [15]. However, to date no oxidation-reduction measurements have been made on any thioredoxin *f* nor on the active site cysteines of any FTR. It is well known that organosulfur derivatives coordinate strongly to many metal surfaces and that they or their reaction products can react directly with the electrode material [16,17]. Therefore, in order to establish reversible electron transfer conditions at the electrode/solution interface, we have used lipid bilayer modification of the electrode surface. In our previous studies [14], we have demonstrated that such electrodes produce well-defined, quasi-reversible and diffusion-controlled cyclic voltammograms with organic and protein disulfides. Below we report the results of direct electrochemical determinations of midpoint potential values for the disulfide/dithiol couples of spinach thioredoxins *f* and *m* and of spinach FTR. The cyclic voltammetry protocol used for these disulfide/dithiol measurements, a technique which has previously been demonstrated to be effective for oxidation-reduction measurements on other thioredoxins and on glutathione [14], has also been used to determine the midpoint potential value for a high-potential center we have detected in spinach FTR. The high-potential FTR component is likely to be the previously detected [4Fe-4S] cluster [18].

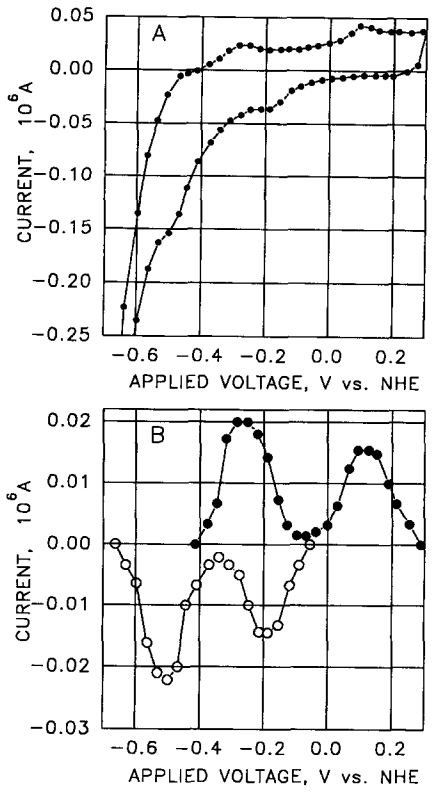

Fig. 1. Cyclic voltammogram of ferredoxin:thioredoxin reductase in the region from −0.65 V to +0.25 V. (A) The reaction mixture contained 78 μM FTR in 25 mM potassium phosphate buffer (pH 6.8) containing 25 mM $NaClO_4$. The voltammogram was obtained at a scan rate of 20 mV/s using a gold electrode modified with a phosphatidylcholine bilayer. A lipid concentration in the membrane-forming solution of 2.5 mg/ml was used. The potentials are reported with respect to the normal hydrogen electrode. (B) Current-voltage reduction (open circles) and oxidation (closed circles) curves obtained from (A) after background subtraction.

2. Materials and methods

Spinach thioredoxins *m* [19] and *f* [20], FTR [4] and ferredoxin [21] were prepared as described previously. The active site cysteines of FTR were alkylated by treatment with NEM (*N*-ethylmaleimide) as described previously [22].

Self-assembled lipid bilayer membranes were deposited on a gold electrode from a membrane-forming solution containing egg lecithin (PC, phosphatidylcholine, Sigma Chemical Corporation) at a concentration of 2.5 mg/ml in squalene (Fluka Chemie)/butanol (1:3, v/v) as previously described [23]. A 0.5 mm diameter gold wire (Aldrich Chemical Co.) with a Teflon sleeve of 0.52 mm inner diameter was used as a working electrode and a saturated Ag/AgCl electrode was used as the reference electrode. All solutions were degassed with high purity argon before use. Cyclic voltammetry was performed [24] and data were analyzed [25] as described previously. All potential values are reported vs. the standard hydrogen electrode.

3. Results and discussion

Fig. 1A shows a cyclic voltammogram of spinach FTR at a self-assembled egg PC bilayer on a gold electrode. Very similar responses were observed for thioredoxins *f* and *m* (data not shown). As has been shown previously for glutathione and the thioredoxins from *E. coli* and T4 [14], all the voltammograms obtained with the three spinach proteins revealed two sets of electrochemical waves, which can be seen better after background subtraction. This is shown for FTR in Fig. 1B. This observation indicates that, as is the case for other disulfide/dithiol systems studied using this technique [14], reduction of spinach thioredoxins *f* and *m* and of the active site of spinach FTRTable 1 occurs via two separate one-electron processes. Table 2 1 summarizes the electrochemical parameters (peak separa-

Table 1
Electrochemical parameters of thioredoxin *m* (Td-*m*), thioredoxin *f* (Td-*f*), and native ferredoxin:thioredoxin reductase (FTR)

	Td-*m*	Td-*f*	FTR
$\Delta E^{(I)}$ (mV)	355	365	300
$\Delta E^{(II)}$ (mV)	135	115	245
$E^{(I)}_{1/2}$ (mV)	− 40 ± 10	− 20 ± 10	− 60 ± 10
$E^{(II)}_{1/2}$ (mV)	−380 ± 10	−400 ± 10	−400 ± 10

All parameters were measured at a potential scan rate of 20 mV/s at a gold electrode modified with a phospatidylcholine bilayer. All voltages are reported with respect to the normal hydrogen electrode. The FTR data were taken from the experiment of Fig. 1A. The values for thioredoxin *m* and thioredoxin *f* were obtained using 150 μM protein in 25 mM Tris-Cl buffer (pH 7.0) containing 25 mM $NaClO_4$ and 200 μM protein in 20 mM triethanolamine-acetate buffer (pH 7.0) containing 150 mM sodium acetate and 2% (v/v) dimethylsulfoxide, respectively.

tions: ΔE^{I}, ΔE^{II}; and half-wave potentials: $E^{I}_{1/2}$, $E^{II}_{1/2}$) for both waves of the voltammograms. The values of the separation of the oxidation and reduction peak currents (ΔE in Table 1) indicate that, as in previous measurements [14], both electrochemical waves are most likely due to slow quasi-reversible electron transfer processes [26,27]. The electrochemical rate of the first wave, i.e., the one occurring at higher potential, is smaller than the second one for all the proteins used in this study (Fig. 1B). Complete reduction of these disulfides requires the transfer of two electrons from the working electrode. One can calculate a thermodynamic value for the half-wave potential of such a two-electron process ($E_{1/2}$) from the average of the $E^{I}_{1/2}$ and $E^{II}_{1/2}$ values given in Table 1, and the $E_{1/2}$ values of the three spinach proteins investigated in this study are summarized in Table 2. The values for spinach thioredoxins *f* and *m* are slightly more positive than those previously reported for other thioredoxins (see above). As the experiments reported herein represent the first determination of a midpoint potential for the active site of any FTR, it is not possible at this time to compare the −230 mV value measured for spinach FTR to values obtained for other FTR's.

It has been known for some time that spinach FTR contains a single [4Fe-4S] cluster [1,2]. Early electron paramagnetic resonance (EPR) investigations demonstrated that the protein was EPR silent when the cluster was in the reduced form [18]. When the protein was oxidized by the addition of ferricyanide, an EPR signal could be detected with a spectrum that resembled that of HiPIP [18]. Oxidation-reduction titrations indicated a midpoint potential of +410 mV for the cluster in FTR isolated from the cyanobacterium *Nostoc muscorum* [18]. It was naturally of interest to determine whether such a high-potential redox center could be detected in spinach FTR by cyclic voltammetry. Fig. 2A shows a cyclic voltammogram for spinach FTR in the high potential region and Fig. 2B shows current-voltage oxidation and reduction curves obtained from the data in Fig. 2A, after background subtraction. An $E_{1/2}$ value of +340 ± 30 mV could be calculated for this one-electron redox center (Table 2).

The +340 mV value for the midpoint potential of the the high-potential component detected in spinach FTR determined by cyclic voltammetry is significantly different, even when the experimental uncertainties in both measurements are taken into consideration, from the +410 mV value for the [4Fe-4S] cluster deduced from EPR spectra of *N. muscorum* FTR samples frozen at defined ambient potentials [18]. However, as the only prosthetic groups known to be present in FTR are the active site disulfide/dithiol and the [4Fe-4S] cluster, it seemed highly likely that the high potential component observed in cyclic voltammograms arises from oxidation and reduction of the [4Fe-4S] cluster. The differences in midpoint potential

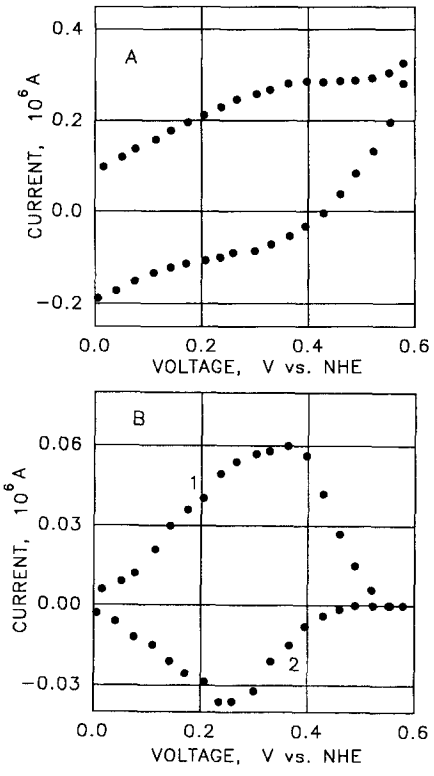

Fig. 2. Cyclic voltammogram of ferredoxin:thioredoxin reductase in the region from 0.0 V to +0.60 V. (A) Reaction conditions were as in Fig. 1A. (B) Current-voltage oxidation (1) and reduction (2) curves obtained from (A) after background subtraction.

Table 2
Half-wave potential values for thioredoxins *m* and *f* and for native and NEM-modified ferredoxin:thioredoxin reductase

	Td-*m*	Td-*f*	FTR	NEM-FTR
$E_{1/2}$ (mV)	−210 ± 10	−210 ± 10	−230 ± 10, +340 ± 30	n.d., +380 ± 20

Values for two-electron processes were calculated from the experimental values of Table 1 for two one-electron steps. All voltages are reported with respect to the normal hydrogen electrode. n.d., not detected.

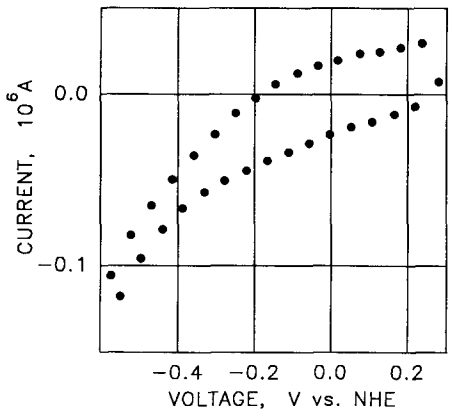

Fig. 3. Cyclic voltammogram of NEM-modified ferredoxin:thioredoxin reductase in the region from from −0.60 V to +0.25 V. Reaction conditions were as in Fig. 1A except that native FTR was replaced by the NEM-modified enzyme.

values may derive from the fact that FTR's isolated from two different species were used for the two measurements and/or from artifacts arising from the cryogenic temperatures required to observe the EPR signals [25]. To provide additional evidence for the assignments of the two components observed in cyclic voltammograms of FTR, the experiments described above were repeated using FTR treated with NEM to alkylate the active-site cysteines [22]. Fig. 3 shows that, in contrast to the results obtained with unmodified FTR (Fig. 1), no low-potential component can be observed in cyclic voltammograms of the NEM-treated enzyme. Fig. 4A shows the cyclic voltammogram observed with the same NEM-modified FTR sample in the high potential region. As can be seen from the current-voltage oxidation and reduction curves shown in Fig. 4B, the high-potential component remains present in NEM-treated FTR, although its midpoint potential appears to have shifted slightly to $+380 \pm 20$ mV (Table 2). However, the experimental uncertainties in the two determinations make it impossible to be confident that the midpoint potential has in fact shifted significantly.

Earlier investigations of the ferredoxin/FTR/thioredoxin system in spinach had resulted in a proposal that electrons flowed from reduced ferredoxin to FTR and then to thioredoxins *f* and *m* [1]. The data obtained in this study, combined with the well-established value of −430 mV for the midpoint potential of ferredoxin [2], indicate that the previously proposed pathway for electron flow is thermodynamically favorable, with FTR having a substantially more positive midpoint potential than that of ferredoxin and a slightly more negative midpoint potential than those of the two chloroplast thioredoxins. What remains puzzling is what role the high-potential [4Fe-4S] cluster could have in such a process.

Complex formation between ferredoxin and ferredoxin:$NADP^+$ reductase (FNR) is known to shift the midpoint potentials of both proteins [2]. As FTR is known to form a high-affinity, electrostatically-stabilized, 1:1 complex with ferredoxin that resembles in some ways the complex [2–4,28] formed between ferredoxin and FNR, it seemed at least possible that complex formation with ferredoxin might shift the midpoint potential of FTR's [4Fe-4S] cluster to a value in the same range as those found for the disulfide/dithiol groups examined in this study. However, cyclic voltammograms of spinach FTR run in the presence of equimolar ferredoxin and at low ionic strength were essentially identical to those obtained in the absence of ferredoxin (data not shown).

It should be pointed out that, although it is difficult to accurately quantitate the relative concentrations of the centers responsible for the high-potential and low-potential features seen in cyclic voltammograms of spinach FTR, it appears that the higher-potential component is present at a significantly lower stoichiometry. This observation raises the possibility that the $[4Fe\text{-}4S]^{2+,3+}$ redox transition observed in the EPR (and probably in the cyclic voltammograms reported above) represents a minor component that is not involved in the electron transfer reaction catalyzed by FTR. This possibility is currently under investigation.

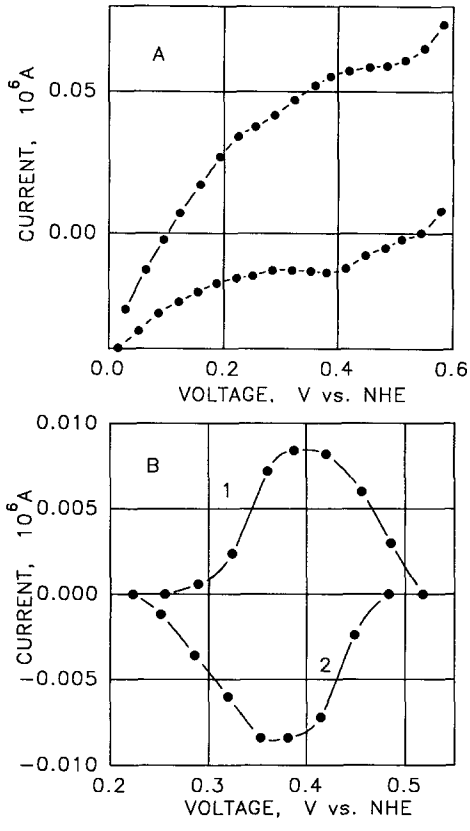

Fig. 4. Cyclic voltammogram of NEM-modified ferredoxin:thioredoxin reductase in the region from 0.0 V to +0.60 V. (A) Reaction conditions were as in Fig. 1A except that NEM-modified FTR, at a concentration of 50 μM in 20 mM triethanolamine-chloride buffer (pH 7.0) containing 40 mM $NaClO_4$, was present instead of the native enzyme. (B) Current-voltage oxidation (1) and reduction (2) curves obtained from (A) after background subtraction.

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