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OXIDATION–REDUCTION PROPERTIES OF PHOTOSYNTHETIC NITRITE REDUCTASE

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1. Introduction

The biological reduction of nitrate to ammonia occurs in two steps. In the first step, nitrate is reduced to nitrite and in the second, nitrite is reduced to ammonia [1]. The conversion of nitrite to ammonia in plants and algae is catalyzed by the enzyme ferredoxin:nitrite oxidoreductase (EC 1.7.7.1), which recently has been shown to contain a heme (siroheme [2–4]) and an iron–sulfur center [3,4]. The enzyme, which consists of a single polypeptide chain of mol. wt 61 000, appears to contain one siroheme and a single iron–sulfur center that contains two irons and two acid-labile sulfides [4].

In this communication we report the oxidation–reduction properties of the electron-transferring prosthetic groups in photosynthetic nitrite reductase. We have used electron paramagnetic resonance (EPR) spectroscopy in conjunction with oxidation–reduction potentiometry to estimate the midpoint potential of these groups.

2. Methods

Ferredoxin : nitrite oxidoreductase was purified according to Vega and Kamin [4] with the exception that chromatography on Bio-Gel A1.5 replaced chromatography on Sephadex G-200 as the final purification step.

Anaerobic oxidation–reduction titrations were performed under an argon atmosphere as described by Dutton [5]. The oxidation–reduction potential was measured with a Metrohm model 103 pH meter and a combination platinum–$Ag/AgCl_2$ electrode (Metrohm EA259) calibrated against a saturated quinhydrone solution, at pH 7.0. Samples were transferred with a Hamilton gas-tight syringe to calibrated EPR tubes made anaerobic by flushing them with argon. Samples were frozen to 77°K prior to EPR analysis at about 15°K. EPR spectra were recorded with a modified JEOL X-band spectrometer which had incorporated into it an AirCo liquid helium cooling system [6,7].

3. Results

Previous optical absorbance measurements provided evidence that the siroheme group of nitrite reductase is the site at which nitrite binds to the enzyme [3,4]. Further support for the conclusion that nitrite interacts with siroheme is found in the EPR spectra of fig.1. The isolated nitrite reductase exhibits EPR signals in the low-field region at g 6.7 and g 5.2. (An additional EPR signal at g 4.3, not shown in fig.1, probably arises from nonspecifically bound ferric ions.) The signals at g 6.7 and g 5.2 are characteristic

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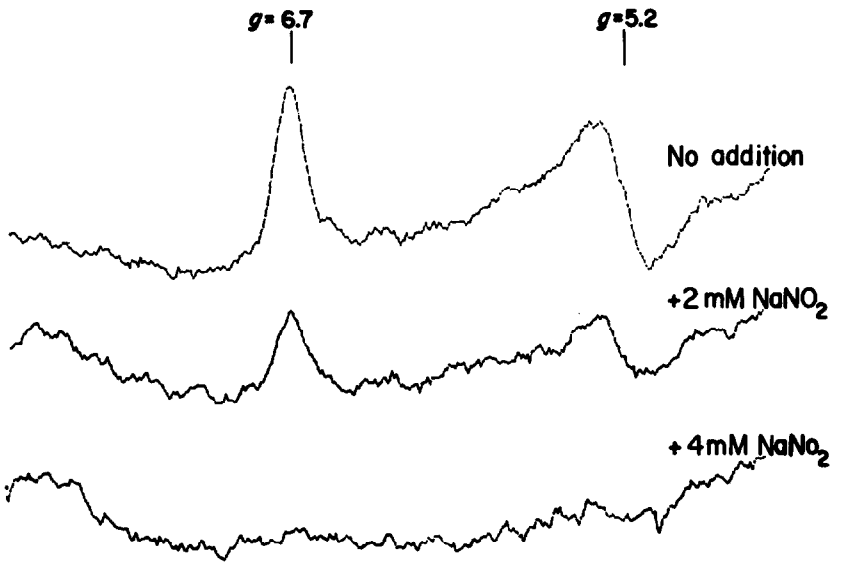

Fig.1. Effect of nitrite on low-field siroheme EPR signals in nitrite reductase. Nitrite reductase (1.1 mg/ml) was incubated with the indicated amount of sodium nitrite for 2 min at 4°C prior to freezing to 77°K. EPR conditions: Frequency, 9.20 GHz; modulation amplitude, 10 G; microwave power, 5 mW; amplifier gain, 320; temperature 13°K; field setting, 1200 ± 500 G.

of a high-spin ferric heme. The latter two signals disappear after the additon of nitrite (fig.1), but the *g* 4.3 signal remains unchanged. A similar loss of high-spin ferric EPR signals was observed after the addition of cyanide, an inhibitor that is competitive with nitrite. No low-spin EPR signals could be detected after the additon of either of these compounds.

Because enzymatic reduction of nitrite is known to be mediated by the bound siroheme [3,4], we investigated the midpoint oxidation–reduction potential of this prosthetic group. The loss of the high-spin EPR signal on ligand binding, a property also observed by Vega and Kamin [4], complicates titrations of the enzyme with sodium dithionite. The use of dithionite inevitably results in the presence of some sulfite, a competitive inhibitor and heme ligand known to cause the disappearance of the high-spin heme signals. Therefore, the disappearance of the *g* 6.7 and *g* 5.2 EPR signals during a reductive titration with dithionite could be the result either of an actual reduction of the ferric heme to the ferrous state or of the formation of a sulfite–heme complex. To avoid these possibilities, we titrated nitrite reductase with reduced methyl

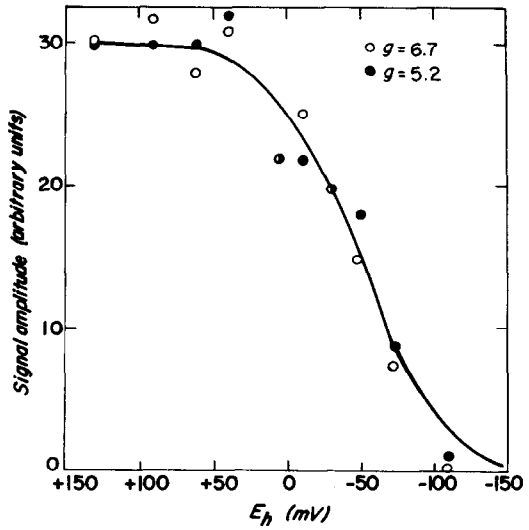

Fig.2. Oxidation–reduction titration of the siroheme EPR signals in nitrite reductase. The reaction mixture contained 100 mM Tris–HCl buffer (pH 7.8), nitrite reductase (1.1 mg/ml) and the following oxidation–reduction mediators at a concentration of 50 μM: Anthraquinone disulfonate, indigosulfonate, indigotetrasulfonate, pyrocyanine, duroquinone, juglone, phenazine ethosulfate, phenazine methosulfate, 1,2-naphthoquinone and diaminodurene. The reductant was 50 mM reduced methyl viologen. EPR conditions were as in fig.1.

viologen (reduced with metallic zinc); we believe that under these conditions the resultant loss of the high-spin ferric heme EPR signal is caused by the reduction of ferric heme rather than by any type of complex formation with the heme. The results of this titration, shown in fig.2, demonstrate that the EPR signals at g 6.7 and g 5.2 show similar dependence on oxidation–reduction potential. The data give a good fit for an $n = 1$ titration with a midpoint oxidation–reduction potential of −50 mV.

An attempt was made to determine the midpoint oxidation–reduction potential of the iron–sulfur center of nitrite reductase. As shown in fig.3, the EPR signal that is characteristic of the reduced iron–sulfur center does not begin to appear until potentials near −500 mV are reached. As the ambient potential is lowered further, the intensity of the signal increases. Because of uncertainty of the titration endpoint (resulting from inability to achieve ambient potentials more electronegative than −560 mV), it was not possible to obtain an exact midpoint potential. The titration was done at pH 9.0, a pH at which the enzyme had approximately half of its maximum activity. Titrations at more alkaline pH values, which would have allowed more electronegative ambient potentials to be

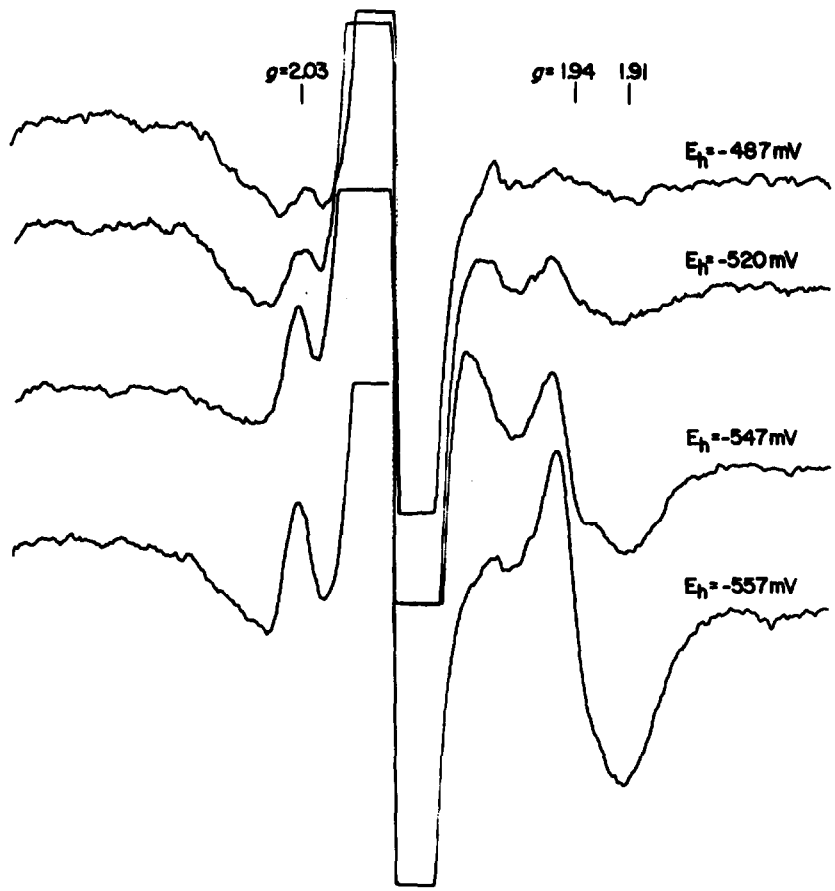

Fig.3. Oxidation–reduction titration of the iron–sulfur center in nitrite reductase. The reaction mixture contained 100 mM glycine buffer (pH 9.0), nitrite reductase (0.75 mg/ml) and the following oxidation–reduction mediators at a concentration of 75 μM: Benzyl viologen, methyl viologen, and triquat. The reductant was 100 mM sodium dithionite (in 0.03 M KOH). EPR conditions: Field setting, 3300 ± 250 G: microwave power, 10 mW; modulation amplitude, 10 G; temperature, 14°K; frequency, 9.20 GHz.

reached, were not possible because of inactivation of the enzyme. From the data in fig.3 it is possible to estimate that the iron–sulfur center of nitrite reductase has a midpoint potential of approximately −550 mV (assuming $n = 1$). In titrations with dithionite as the reductant (required for the generation of an extremely electronegative potential), sulfite is invariably present. Thus, the data shown in fig.3 probably reflect the oxidation–reduction properties of the iron–sulfur center in the sulfite–heme complex rather than in the free enzyme. To check the effect of heme ligands on the potential of the iron–sulfur center, we titrated the iron–sulfur center in the presence of cyanide (5 mV), a ligand known to have much higher affinity for the heme than does sulfite [3]. Results similar to those shown in fig.3 were obtained in this experiment.

4. Discussion

Nitrite reductase appears to function via a mechanism in which nitrite binds to the oxidized siroheme and is then reduced by electrons from the iron–sulfur center [3]. The physiological electron donor for nitrite reductase in plants and algae is known to be reduced chloroplast ferredoxin [1,8]. One conceptual problem raised by our results is how chloroplast ferredoxin (midpoint oxidation–reduction potential −420 mV [9]) can reduce the iron–sulfur center of nitrite reductase (midpoint potential more electronegative than −500 mV). The most probable explanation is that the midpoint potential of the iron–sulfur center under physiological conditions shifts to a more electropositive value. In all likelihood, the value we have measured was obtained under conditions where siroheme is present in an inhibitor (sulfite) complex. The nitrite–heme complex (the natural enzyme–substrate complex) may interact with the iron–sulfur center differently than with the inhibitor–heme complex and alter the potential of the iron–sulfur center. Formation of a complex between reduced ferredoxin and nitrite reductase during the reaction might also be an important factor in controlling the potential of the iron–sulfur center in the enzyme.

We have found that the siroheme in nitrite reductase has a midpoint potential of −50 mV. This is the first reported midpoint potential for siroheme in an enzyme, and it will be of interest to compare this value with that of the siroheme in another enzyme, sulfite reductase [2]. It has been suggested [2,3] that nitrite reductase reduces nitrite to ammonia via enzyme-bound NO and NH_2OH. It is difficult to assess the midpoint potential of het NO_2^-/NO and NH_2OH/NH_3 complexes that may form during catalysis because the species involved would be bound to the siroheme rather than free in solution. Our results do, however, provide a first indication of the thermodynamic properties of this group which functions in enzymatic reduction of nitrite.

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