Detection of Sulfide Release from the Oxygen-sensing [4Fe-4S] Cluster of FNR*

The Escherichia coli FNR protein regulates the transcription of >100 genes in response to environmental O2, thereby coordinating the response to anoxia. Under O2-limiting conditions, FNR binds a [4Fe-4S]2+ cluster through four cysteine residues (Cys20, Cys23, Cys29, Cys122). The acquisition of the [4Fe-4S]2+ cluster converts FNR into the transcriptionally active dimeric form. Upon exposure to O2, the cluster converts to a [2Fe-2S]2+ form, generating FNR monomers that no longer bind DNA with high affinity. The mechanism of the cluster conversion reaction and the nature of the released iron and sulfur are of considerable current interest. Here, we report the application of a novel in vitro method, involving 5,5′-dithiobis-(2-nitrobenzoic acid), for determining the oxidation state of the sulfur atoms released during FNR cluster conversion following the addition of O2. Conversion of [4Fe-4S]2+ to [2Fe-2S]2+ clusters by O2 for both native and reconstituted FNR results in the release of ~2 sulfide ions per [4Fe-4S]2+ cluster. This demonstrates that the reaction between O2 and the [4Fe-4S]2+ cluster does not require sulfide oxidation and hence must entail iron oxidation.

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oxygen-free nitrogen gas at 4 °C, essentially as described by Sutton and Kiley (25). All protein purification and handling was carried out under strictly anaerobic conditions in an anaerobic cabinet (Belle Technology), typically operating at ≤2.0 ppm O₂ by volume, equipped with a specially designed fridge-freezer for anaerobic sample storage and fitted with a liquid nitrogen access port. All buffers were sparged with oxygen-free nitrogen gas for a minimum of 2 h. The purity of isolated FNR was assessed by SDS-PAGE. Full details of the growth conditions and purification of FNR will be published elsewhere.

**Purification of Reconstituted [4Fe-4S] FNR**—Glutathione S-transferase-FNR fusion protein was produced in aerobically grown *E. coli* BL21ADE3 pGS572, as described previously (17). FNR was cleaved from the fusion protein using thrombin and [4Fe-4S] FNR reconstituted *in vitro*, as described previously (17), except that a 1-ml HiTrap heparin column (GE Healthcare) was used (25) in place of a Sephadex G25 column to remove low molecular weight contaminants and to perform buffer exchange.

**Purification of [2Fe-2S] FNR**—An aliquot of native [4Fe-4S] FNR (1 ml, ~119 μM in cluster) in buffer A was removed from the anaerobic cabinet, treated with 500 μl of buffer A containing dissolved atmospheric oxygen, and gently agitated at air for 90 s. The sample was then returned to the anaerobic cabinet. [2Fe-2S] FNR was isolated from cluster breakdown products using a Sephadex G25 column (PD10, GE Healthcare). The protein, iron, and sulfide contents of the sample were determined as described below.

**Quantitative Methods**—FNR protein concentrations were determined using the method of Bradford (Bio-Rad), with bovine serum albumin as the standard (26) and a previously determined correction factor of 0.83 (15). FNR iron content was determined in the following way: 0.1 ml of 21.7% HNO₃ was added to the same volume of protein and incubated at 95 °C for 30 min. Cooled samples were centrifuged to remove any precipitate, treated with 0.6 ml of 7.5% (w/v) ammonium acetate, 0.1 ml of 12.5% (w/v) ascorbic acid, 0.1 ml of 10 mM Ferene, mixed, and incubated at room temperature for 30 min before absorbance at 593 nm was measured. Iron concentrations were determined by reference to a calibration curve generated from Fe³⁺ solutions in the range 0–200 μM, prepared from Spectrostar. standard iron solution (BDH, Lot OC495679), and treated as described above. Acid-labile sulfide was determined according to the method of Beinert (27). Based on the analyses, both native and reconstituted [4Fe-4S]²⁺ FNR samples exhibited ε₄₀₅ nm values of 16,220 ± 135 M⁻¹ cm⁻¹, in close agreement to previously reported values (23). The concentration of dissolved atmospheric oxygen present in buffer solutions was determined by chemical analysis according to the method of Winkler (28).

**Quantitation of Sulfide Released during Cluster Conversion**—Quantitation of sulfide released from the cluster during the [4Fe-4S]²⁺ to [2Fe-2S]²⁺ conversion was carried out using a modified version of the procedure based on DTNB (Ellman’s reagent) reported by Nashef and colleagues (29). Briefly, [4Fe-4S] FNR (~5–10 μM) was treated with DTNB (~200 μM) under anaerobic conditions and incubated at room temperature for 2 min prior to measurement of absorbance at 412 nm (30). The [4Fe-4S]²⁺ to [2Fe-2S]²⁺ cluster transition was subsequently induced by injecting an aliquot of buffer containing dissolved atmospheric oxygen (~40 μM final concentration). Absorbance at 412 nm was measured again after an incubation period of 12 min at room temperature (sufficient time for the reaction (ΔA₁₁₁₂ nm) to plateau under the given conditions (data not shown)). The reactive thiol content of [2Fe-2S] FNR was measured as absorbance changes at 412 nm following the anaerobic addition of DTNB (~200 μM) and incubation for 2 min at room temperature.

Reactive thiol and free sulfide concentrations were calculated by determining the concentration of released TNB anion, using an ε₄₁₁₂ value of 14,151 M⁻¹ cm⁻¹ in buffer A at pH 6.8, 12,344 M⁻¹ cm⁻¹ in buffer B at pH 7.5, or 14,611 M⁻¹ cm⁻¹ in buffer B containing 6 mM guanidine HCl (see below). ΔA values at 412 nm were corrected for the changes at 412 nm, which were due only to the [4Fe-4S]²⁺ to [2Fe-2S]²⁺ conversion. To determine whether the liberation of the TNB anions was due, in part, to the generation of superoxide or hydrogen peroxide, experiments were repeated in the presence of catalase (268 units) and superoxide dismutase (35 units). Iron released during the conversion was determined by adding Ferene (100 μM) and ascorbate (600 μM), to generate [Fe(II)(Ferene)]³⁻, and by incubating at room temperature for 2 min prior to measurement of absorbance at 593 nm. Adventitiously bound iron was determined by treating an anaerobic [4Fe-4S] FNR sample with Ferene and ascorbate as described above. Experiments were performed in two different buffer systems: 10 mM potassium phosphate, 400 mM KCl, 10% (v/v) glycerol, pH 6.8 (buffer A), and 25 mM HEPES, 2.5 mM CaCl₂, 100 mM NaCl, 100 mM NaN₃, pH 7.5 (buffer B).

We verified that the [4Fe-4S]²⁺ cluster was stable to the presence of DTNB and Ferene in both buffer systems tested under anaerobic conditions, at least for the duration of the experiment (data not shown). The response of DTNB to sulfide was calibrated using a standard solution of Na₂S prepared as described by Beinert (27) and verified by iodometric titration as described by Vogel (28). 0.1 ml of Na₂S was added to 2 ml of buffer A containing excess DTNB (2.4 mM) and absorbance at 412 nm measured after 2 min. The amount of TNB anion produced was calculated using a ε₄₁₁₂ value of 14,151 M⁻¹ cm⁻¹. To assess the effects of protein and Fe²⁺, DTNB reactions were repeated in the presence of lysozyme (0.8 mg/ml) and (NH₄)₂Fe(SO₄)₂ (117 μM). Cysteine, rather than Na₂S, was used for the Fe²⁺ control experiment due to the poor solubility of FeS. All determinations were carried out in triplicate. Absorbance measurements were made with a Jasco V550 UV-visible spectrophotometer.

**RESULTS AND DISCUSSION**

Since the initial report by Ellman (31) on the thiol-specific reactions of DTNB, the reagent has been used extensively as a means for the quantitation of thiol groups in proteins. However, DTNB may also be used as a colorimetric reagent, as reported by Nashef et al. (29), for the determination of sulfide ions, see Scheme 1. We have suitably modified the published method (29) to quantify sulfide released from FNR during [4Fe-4S]²⁺ to [2Fe-2S]²⁺ cluster conversion.
The reaction of DTNB with sulfide ion was calibrated against standard Na₂S solutions, as described under “Experimental Procedures.” These data (data not shown) confirmed that sulfide ions react stoichiometrically with DTNB to yield two TNB anions, as reported previously (29). We also verified that neither the presence of protein nor Fe²⁺ interferes with the DTNB reaction. From this we concluded that the assay can be used to measure the concentration of sulfide ion in solution.

The addition of excess DTNB to [4Fe-4S] FNR under anaerobic conditions causes spectral changes due to the release of TNB anions (Fig. 1A). These arise from the reaction of DTNB with free protein thiol. FNR contains five cysteine residues of which four (Cys²⁰, Cys²³, Cys²⁹, Cys¹²²) ligate the [4Fe-4S] cluster. Hence Cys¹⁶ should be available for reaction with a modifying reagent such as DTNB, see Scheme 2. In addition, samples of reconstituted and native FNR are not 100% replete with [4Fe-4S] cluster. There is a component of cluster-free (apo-) protein typically ranging from 15 to 30% and 23 to 43% for reconstituted and native FNR, respectively. All five cysteines of apo-FNR are potentially available for reaction with DTNB. However, it has been demonstrated recently (32) that only four out of the five cysteines present in apo-FNR are reactive toward thiol-specific modifying reagents under anaerobic conditions in the presence of denaturants. We have found that the apo-FNR content of samples contains up to three thiols available for modification (see Table 1) depending upon the method used for purification. Under anaerobic denaturing conditions (buffer B containing 6 M guanidine HCl) we have found 4.7 (± 0.4) and 4.1 (± 0.5) thiols available for modification per monomer in reconstituted and native FNR, respectively (data not shown), consistent with the observations of Achebach and colleagues (32).

The introduction of oxygen causes further substantial changes in the UV-visible spectrum of the FNR DTNB mix due to TNB anion release (Fig. 1B). Since DTNB is inert to oxygen (30), the observed reaction is due to changes in FNR following cluster conversion.

To determine the amount of sulfide ion released during FNR cluster conversion, a correction to the A₄₁₂ nm value was made to account for the spectral changes arising from the cluster conversion [4Fe-4S] to [2Fe-2S] (Fig. 1B). The [2Fe-2S] clusters are believed to have the same cysteine ligands as the [4Fe-4S]²⁺ clusters (13). To confirm that cluster conversion does not expose any of the [4Fe-4S]²⁺ cysteine ligands to reaction with thiol modification reagents, [2Fe-2S] FNR was isolated, as described under “Experimental Procedures.” Reaction of an aliquot of 5.2 μM [2Fe-2S] FNR (contain-
ing 5.2 μM protein, 10.9 μM iron, and 10.2 μM sulfide) with DTNB resulted in a TNB anion concentration of 5.7 μM, indicating the modification of 1.1 (±0.1) thiols per [2Fe-2S] FNR. In the absence of apo-FNR, this is consistent with the modification of Cys^{16} in each [2Fe-2S] FNR molecule. This supports the conclusion that Cys^{20}, Cys^{23}, Cys^{29}, and Cys^{122} are the ligands that bind both the [4Fe-4S]^{2+} and [2Fe-2S]^{2+} clusters (13). Hence the observed increase in absorbance at 412 nm is not due to further thiol modification. Instead, we ascribe it to the reaction of DTNB with sulfide ions released during cluster conversion.

A series of experiments on both native and reconstituted [4Fe-4S] FNR, in two different buffer systems, all resulted in the detection of ~2 sulfide ions per cluster (see Table 1). In addition to two sulfide ions, two iron ions should be released during cluster conversion. We determined iron released during conversion by the introduction of Ferene and ascorbate following cluster conversion. We determined iron released during conversion to two sulfide ions, two iron ions should be released during 

\[
4Fe-4S^{2+} + O_2 \rightarrow [2Fe-2S]^{2+} + Fe^{3+} + 2Fe^{2+} + 2S^{2-} + O_2^- \tag{Eq. 2}
\]

Indeed, such a mixture of iron oxidation states has been observed by Khoroshilova et al. (13, 16). Furthermore, we cannot rule out the possibility that the hydrogen peroxide detected following reaction of [4Fe-4S] FNR with oxygen (17) originates from the dismutation of superoxide. Importantly, it has been reported that superoxide can reduce DTNB to TNB species (33). Therefore, the sulfide release experiments were repeated in the presence of catalase and superoxide dismutase. No specific effects attributable to superoxide (or hydrogen peroxide) were detected (Table 1).

Recently Sutton et al. (23) proposed an alternative mechanism of [4Fe-4S]^{2+} to [2Fe-2S]^{2+} conversion that proceeds via the oxidation of cluster S^{2-} ions (here termed sulfide oxidation). This was based on the observation that, in the presence of the strong Fe^{3+} chelator, Ferene, two Fe^{2+} ions were detected per cluster following reaction with oxygen. This leads to the possibility of a four-electron reduction of oxygen to water at the expense of cluster sulfide (see Equation 3) (23).

\[
4Fe-4S^{2+} + O_2 + 4H^+ \rightarrow [2Fe-2S]^{2+} + 2Fe^{2+} + 2S^{2-} + 2H_2O \tag{Eq. 3}
\]

Previously we have proposed a mechanism for FNR cluster conversion based on the observation of a [3Fe-4S]^{1+} intermediate cluster together with the detection of hydrogen peroxide generated during cluster conversion (Ref. 17; see Equation 4). Note that the [3Fe-4S]^{1+} cluster contains three Fe^{3+} ions.

\[
[4Fe-4S]^{2+} + O_2 + 2H^+ \rightarrow [3Fe-4S]^{1+} + Fe^{3+} + H_2O \rightarrow [2Fe-2S]^{2+} + Fe^{3+} + 2S^{2-} \tag{Eq. 4}
\]

This mechanism results in an overall two electron oxidation reaction, in which both Fe^{2+} ions present in the [4Fe-4S] cluster

**TABLE 1**

| Sample no. | Anaerobic | After addition of oxygen (~ 40 μM) |
|------------|-----------|-----------------------------------|
|            | Protein | Apo-protein | [4Fe-4S]| TNB* | Thiol/apo* | Total TNB | ΔTNB | Released Sulfide | Average released per [4Fe-4S] |
|            | μM | μM | μM | μM | μM | μM | μM | μM | μM | μM | μM |
| 1 | 6.4 | 1.5 | 4.9 | 7.1 | 1.5 | 27.3 | 20.2 | 10.1 | 9.8 | 2.1 (±0.5) | 1.9 (±0.2) |
| 2 | 10.9 | 4.7 | 6.2 | 11.4 | 1.1 | 34.7 | 23.3 | 11.7 | 11.0 | 1.8 (±0.2) | 1.8 (±0.1) |
| 3 | 8.0 | 2.4 | 5.6 | 10.4 | 2.0 | 32.4 | 22.0 | 11.0 | 11.8 | 1.9 (±0.2) | 2.1 (±0.3) |
| 4 | 6.9 | 2.1 | 4.8 | 11.3 | 3.1 | 29.8 | 18.5 | 9.2 | 9.6 | 2.1 (±0.3) | 2.0 (±0.2) |
| 5 | 5.9 | 0.9 | 5.0 | 7.7 | 3.0 | 24.9 | 17.2 | 8.6 | 9.4 | 1.9 (±0.2) | 2.0 (±0.2) |

*a DTNB was added to the as-isolated protein under non-denaturing anaerobic conditions. The concentration of TNB anion detected corresponds to the modification of C16 in each protein molecule, in addition to the modification of any available cysteine residues in apo-FNR.

*b C16 of [4Fe-4S] FNR is assumed to react stoichiometrically with DTNB. The remaining TNB, therefore, is assumed to originate from apo-FNR.

*c One sulfide ion reacts with DTNB to yield two TNB anions (29).

*d Values were corrected for adventitiously bound iron (see "Experimental Procedures").

*e The assay was repeated in triplicate per FNR preparation.

f The sample contained catalase (268 units) and superoxide dismutase (35 units).
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are oxidized to Fe$^{3+}$ ions (see Equation 5).

\[
[4\text{Fe}-4\text{S}]^{2+} + \text{O}_2 + \text{2H}^+ \rightarrow [2\text{Fe}-2\text{S}]^{2+} + 2\text{Fe}^{3+} + 2\text{S}^{2-} + \text{H}_2\text{O}_2 \quad \text{(Eq. 5)}
\]

To reconcile the apparent inconsistencies in observations by different workers (compare Equations 3 and 5), Sutton et al. (23) suggested that [4Fe-4S] FNR prepared via in vitro reconstitution, as described by Crack et al. (17), reacts in a significantly different manner to the native [4Fe-4S]$^{2+}$ FNR following exposure to oxygen. Here, we have used both in vitro reconstituted and native [4Fe-4S] FNR and find no significant differences in the behavior of the proteins (Table 1). This suggests that the same chemistry occurs in vitro upon exposure to oxygen, regardless of whether [4Fe-4S] FNR is generated in vivo or in vitro.

In conclusion, our results demonstrate that two sulfide ions are ejected from the FNR [4Fe-4S]$^{2+}$ cluster in response to oxygen, in vitro, irrespective of whether the [4Fe-4S] FNR is reconstituted or native protein. This is consistent with the idea that the reaction between oxygen and the cluster occurs via metal oxidation, rather than via a sulfide oxidation pathway. The method of sulfide ion determination reported here might be more widely applicable to other studies of iron-sulfur systems that involve cluster conversion or disassembly.

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