Stimulation of Fe–S cluster insertion into apoFNR by *Escherichia coli* glutaredoxins 1, 2 and 3 in vitro

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Abstract The oxygen sensor fumarate nitrate reductase regulator (FNR) of *Escherichia coli* contains in the active (anaerobic) state a $[4Fe–4S]^{2+}$ cluster which is lost after exposure to O_2. In aerobically prepared apoFNR, or in FNR obtained by treatment of [4Fe–4S] · FNR with O_2 in vitro, intramolecular cysteine disulfides are found, including the cysteine residues which serve as ligands for the Fe–S cluster. It is shown here that the reconstitution of [4Fe–4S] · FNR from this form of aerobic apoFNR was preceded by a long lag phase when glutathione was used as the reducing agent. Addition of *E. coli* glutaredoxins (Grx) 1, 2 or 3 decreased the lag phase greatly and stimulated the reconstitution rate slightly (about twofold). Reconstitution of anaerobically prepared apoFNR, which has a lower cysteine disulfide content, showed only a short lag phase, which further decreased in the presence of Grx. It is concluded that in the lag phase the cysteine disulfides of apoFNR become reduced for the incorporation of the [4Fe–4S] cluster and that this reaction is stimulated by Grx. Thioredoxin (Trx) 1 showed no stimulation of FNR reconstitution in vitro. It is suggested that the function of Grx might be of significance for the insertion of FeS cluster in proteins containing disulfides.

Keywords: Glutaredoxin; Thioredoxin; FNR; Oxygen sensor; Disulfide reductase; Glutathione

1. Introduction

The fumarate nitrate reductase regulator (FNR) of *Escherichia coli* controls the expression of a large number of genes in response to O_2 availability (for reviews compare [1–4]). Under anoxic conditions FNR is a dimer carrying a $[4Fe–4S]^{2+}$ cluster per monomer, which is liganded by four cysteine residues from the total of five cysteine residues of the protein [5–9]. In the presence of O_2, [4Fe–4S] · FNR is converted to [2Fe–2S] · FNR which is no longer active in gene regulation [6,7,10–12]. After extended exposure to O_2, the [2Fe–2S] cluster disintegrates, and apoFNR devoid of Fe and sulfide is formed. ApoFNR prepared under anoxic conditions contains low amounts of disulfides, and incubation of apoFNR or of reconstituted [4Fe–4S] · FNR under oxic conditions causes formation of apoFNR with significantly increased disulfide contents [13].

[4Fe–4S] · FNR can be reconstituted in vitro from aerobic or anaerobic apoFNR [7,11,14,15]. Reconstitution has been achieved by an artificial Fe–S cluster biosynthetic system using Fe(II), cysteine and cysteine desulfurase (from *Azotobacter vinelandii*, $NifS_{Av}$ or from *E. coli*, IscS enzymes). Cysteine desulfurase produces sulfide from cysteine in the presence of a reducing agent such as dithiothreitol [16–18]. In vivo, additional proteins and factors are required. Thus, the FeS cluster is preformed on a scaffold protein (IscU) in a process requiring reducing equivalents and then inserted into the FeS cluster protein [19]. Incorporation and liganding of the FeS-cluster into apoFNR requires ligands Cys20, Cys23, Cys29 and Cys122 in the reduced state. This raises the question whether protein disulfide reductases such as glutaredoxin (Grx) or thioredoxin (Trx) [20] support incorporation of the FeS cluster by transferring cysteine disulfides of apoFNR to the thiol state. Both disulfide reductase systems in principle could serve this function.

In vivo, the presence of five alternative protein disulfide reductases in *E. coli* (Grx1, 2 and 3; Trx1 and 2) complicates studies on the significance of protein disulfide reductases for FeS cluster insertion. Multiple mutations in the *grx* or *trx* genes show growth deficiencies, which complicates in vivo studies and interpretation of the results. Therefore, for testing a potential role of the protein disulfide reductases in the formation of [4Fe–4S] · FNR, stimulation of [4Fe–4S] · FNR reconstitution by Grx or Trx was analysed in vitro. *E. coli* contains at least three different Grx (Grx1, Grx2 and Grx3) and two Trx (Trx1 and Trx2) which could take over this role. The Grx isoforms Grx1, Grx2 and Grx3 (encoded by the *grxA*, *grxB* and *grxC* genes) have similar functions and can replace each other in part [20]. With the exception of Trx2, each of the five protein disulfide reductases was tested for its effect on FNR reconstitution. In this way, it was possible to identify protein disulfide reductases which are able to stimulate [4Fe–4S] · FNR reconstitution in vitro.

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Abbreviations: FNR, fumarate nitrate reductase regulator; Grx, glutaredoxin; $NifS_{Av}$, cysteine desulfurase from *Azotobacter vinelandii*; IscS, cysteine desulfurase from *E. coli*; Trx, thioredoxin

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2. Materials and methods

2.1. Isolation of FNR, $NifS_{Av}$, Grx and Trx1

For overexpression and purification of FNR under aerobic and anaerobic conditions, *E. coli* CAG627pMW68 encoding an inducible GST′-FNR fusion protein was used [15]. For isolation under anaerobic conditions, GST′-FNR bound to the glutathione–Sepharose column [15] was cleaved with thrombin, and FNR devoid of the protein tag was eluted in anoxic Tris–HCl buffer (50 mM at pH 7.6) without using glutathione. All steps were performed in an anaerobic cabinet. For the isolation of FNR under aerobic conditions, growth and isolation were performed under aerobic conditions with oxic buffers. $NifS_{Av}$, Trx1, Grx1, Grx2, Grx3 and Grx2C12S were isolated as described [15,16, 21–23].

2.2. Reconstitution of [4Fe–4S] · FNR from apoFNR

[4Fe–4S] · FNR was reconstituted in buffer containing apoFNR (6.5–20 μM), 0.3 mM $(NH_4)_2Fe(SO_4)_2$, 1 or 2 mM cysteine, $NifS_{Av}$ and glutathione (1 mM) under anoxic conditions [15]. Where indicated, Grx, NADPH (0.27 mM) and yeast glutathione reductase (6 μg/ml), or Trx1, NADPH (0.27 mM) and Trx reductase (0.35 μM), were included in addition. Reconstitution and cluster formation depended on the presence of apoFNR [15] and was followed at 420 nm for the FeS cluster formation relative to the protein content at 280 nm by measuring an optical spectrum (280–550 nm) every 5 or 10 min in a diode array spectrometer (Zeiss S10 with Winaspect software) [15].

2.3. Other methods

Sulfide formation from cysteine by $NifS_{Av}$ was measured in anoxic solutions in the anaerobic cabinet by the formation of FeS in an anoxic buffer containing 2 mM cysteine, 0.3 mM $(NH_4)_2Fe(SO_4)_2$, or by sulfide formation by the methylene blue assay [16,24].

3. Results

3.1. Stimulation of FNR reconstitution by Grx2

Grx2, which is found in high concentrations (about 5 μM) in *E. coli* [25,26], was tested for the effect on FNR reconstitution in vitro. Glutathione was used as a reducing agent, since dithiothreitol, which is commonly used in vitro as the reducing agent for FNR reconstitution, is an artificial reducing agent which reduces non-specifically disulfides of proteins. Reconstitution was tested in a minimal Fe–S cluster biosynthetic system under anoxic conditions consisting of apoFNR, Fe(II), cysteine as a source for sulfur, and the $NifS_{Av}$. This system allows measuring the capacity of apoFNR as a receptor for a [4Fe–4S] cluster [7,11,14,15]. The incorporation of the [4Fe–4S] cluster in FNR was followed by the increase in absorption at 420 nm and the appearance of a bright yellow-brownish colour, which is typical for the [4Fe–4S] cluster in FNR. Reconstitution of aerobically prepared apoFNR, which contains about 2.8 mol cysteine disulfide/mol FNR, started after a long lag phase (Fig. 1A). Inclusion of Grx2 caused a significant decrease in the lag phase. With 5 μM Grx2, the lag phase was largely decreased and the reconstitution rate was increased about threefold. The amounts of reconstituted [4Fe–4S] · FNR were similar in the presence and absence of Grx2. The absorption spectra of FNR reconstituted in the presence of Grx2 showed the same spectral characteristics as [4Fe–4S] · FNR reconstituted without Grx2 (not shown).

Anaerobically prepared apoFNR responded in a different way (Fig. 1B). In the absence of Grx, the formation of the [4Fe–4S] cluster started from the beginning, but with slow rates, which rised with increasing reaction time. In the presence of 5 μM Grx2 on the other hand, the reconstitution started from the beginning without lag phase and high rate. In some experiments

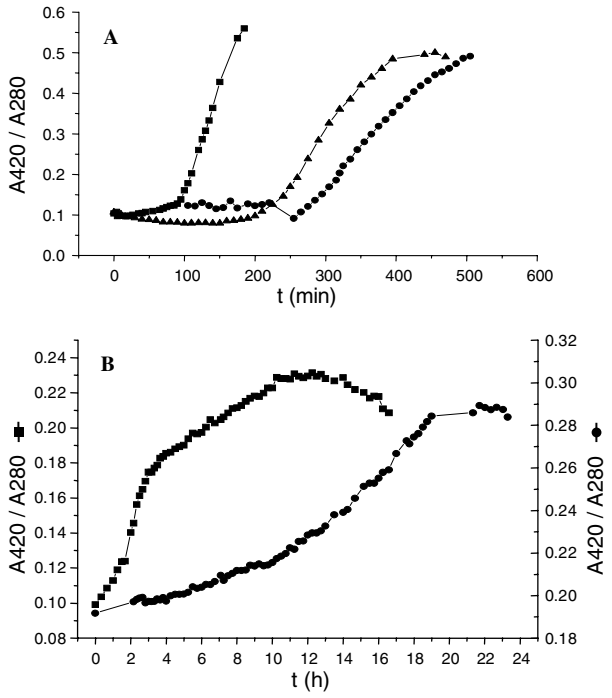

Fig. 1. Effect of Grx2 on the reconstitution of aerobically (A) and anaerobically (B) prepared apoFNR. Aerobically or anaerobically prepared apoFNR (20 μM) was incubated under anoxic conditions with $NifS_{Av}$, Fe(II), cysteine, low amounts of glutathione (1 mM) and various amounts of Grx2 (-●-, 0 μM; -▲-, 1 μM; -■-, 5 μM) plus NADPH plus glutathione reductase. Formation of [4Fe–4S] FNR was followed spectroscopically at 420 and 280 nm.

(Fig. 1B), the reconstitution rate was not constant over the complete experiment which could be due to other factors involved in the reconstitution. Aerobic as well as anaerobic apoFNR reconstituted in this way were capable of specific binding to promoter DNA in a restriction protection assay [15].

When the same reconstitution experiments were performed with dithiothreitol, which is capable of efficient and unspecific reduction of cysteine disulfides, there was no extended lag phase before onset of reconstitution, even when aerobically prepared apoFNR was used. The rate of [4Fe–4S] cluster formation was by factors of 3–5 higher compared to the rate with glutathione [15]. Under these conditions, addition of Grx2 showed no stimulation of the [4Fe–4S] cluster formation (not shown).

3.2. Effects of Grx1 and Grx3 on the stimulation of apoFNR reconstitution

The effects of the other Grx of *E. coli* (Grx1 and Grx3) on apoFNR reconstitution were tested by applying cellular concentrations of the Grx, which differ considerably for the various isoforms [20,22,25–27]. Grx1 caused a decrease in the lag phase before onset of reconstitution of aerobic apoFNR, too (Table 1). Supplying a mixture of Grx1, 2 and 3 resulted in nearly complete loss of the lag phase for aerobic apoFNR, suggesting that the effects of the Grx were additive (Table 1). In addition, each of the Grx caused an up to threefold stimulation of the reconstitution rate. The mixture of the Grx, or higher concentrations, caused no further increase in the reconstitution rate compared to the effect of the single additions (Table 1).

Table 1
Reconstitution of apoFNR and effects of Grx1, 2 or 3 in vitro

Condition	Reconstitution rate (nmol FNR/min)	Lag phase (min)
Aerobic apoFNR		
GSH (1 mM)	0.03	300
GSH (1 mM) + Grx1 (0.2 μM)	0.07	⩽300
GSH (1 mM) + Grx2 (5 μM)	0.09	<100
GSH (1 mM) + Grx3 (2.5 μM)	0.06	ND
GSH + Grx1 (0.2 μM) + Grx2 (5 μM) + Grx3 (2.5 μM)	0.08	<20
GSH (1 mM) + Grx2C12S (5 μM)	0.05	100
Anaerobic apoFNR		
GSH (1 mM)	0.02	ND
GSH (1 mM) + Grx2 (5 μM)	0.04	<20

Reconstitution of apoFNR (20 μM) was performed with 1 mM glutathione as the reducing agent and by the addition of the Grx (Grx1, 2 and 3) as shown in Fig. 1. The rates of reconstitution (maximal rates) and the lag phases were estimated from kinetic experiments (see Fig. 1). ND, not determined; GSH, reduced glutathione.

Grx reduce their substrates by the use of the two active site cysteine residues (CPYC) or a monothiol mechanism employing the N-terminal cysteine and reduced glutathione. The former mechanism can reduce both protein disulfides and protein–glutathione mixed disulfides (protein-SG) [28]. The monothiol mechanism is used for reducing mixed disulfides between proteins and glutathione at the expense of mixed disulfide formation between Grx and glutathione (protein-S-SG + Grx-SH → protein-SH + Grx-S-SG [29,30]). Therefore, the monothiol mutant Grx2C12S of Grx2 [20,21] was tested for its effect on FNR reconstitution. Grx2C12S stimulated reconstitution of [4Fe–4S] · FNR from aerobic apoFNR with glutathione as the reducing agent only slightly (Table 1). The effect on the lag phase before onset of reconstitution, however, was similar to that of wild-type Grx2.

3.3. Effect of Grx on sulfide formation by $NifS_{Av}$

$NifS_{Av}$ or IscS catalyse desulfuration of cysteine, and by reduction of a persulfide intermediate in $NifS_{Av}$, sulfide is released for the formation of the FeS cluster in the protein. The effect of Grx on sulfide formation by cysteine desulfurase was tested by the methylene blue assay, or by the formation of free FeS in the presence of Fe(II) in solution in the absence of apoFNR. The precipitation of FeS is a direct measure for the sulfide formation. Sulfide formation by $NifS_{Av}$, as measured with the methylene blue assay (14 U/g), increased only 20% or less by the addition of Grx1 when glutathione or dithiothreitol was used as the reducing agents (Fig. 2). FeS formation by cysteine desulfurase was also not stimulated by the presence of Grx1 (not shown). Therefore, the stimulation of [4Fe–4S] · FNR formation by Grx, in particular the decrease of the lag phase before reconstitution, has to be caused by effects of Grx on apoFNR, and not on $NifS_{Av}$ stimulation.

3.4. Lack of stimulation of FNR reconstitution by Trx1

The effect of Trx1 on the formation of [4Fe–4S] · FNR from aerobic apoFNR was tested in vitro similar to the experiments performed with the Grx. Trx1, which was reduced by Trx reductase and NADPH, was present in concentrations up to 5 μM in the reconstitution assay. Trx1 concentrations of 5 μM are close to the concentration within the bacteria [31]. However, neither low nor high concentrations of Trx1 caused stimulation of [4Fe–4S] · FNR reconstitution, or of the lag phase before onset of reconstitution (not shown). Trx1 was also not able to stimulate the activity of the $NifS_{Av}$ when tested for the formation of sulfide by the FeS precipitation assay in an experiment performed similar to that in Fig. 2 with Grx. Therefore, Trx1 is not able to stimulate reconstitution of [4Fe–4S] · FNR, and it is assumed that the isoform Trx2 behaves similar.

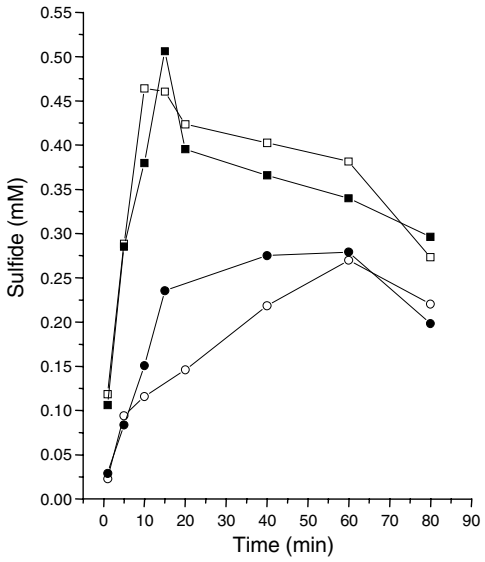

Fig. 2. Sulfide formation by $NifS_{Av}$ under anoxic conditions in the presence and absence of Grx1. $NifS_{Av}$ was incubated under anaerobic conditions with cysteine and glutathione (-●-) or dithiothreitol (-■-) and without (open symbols) or with (closed symbols) Grx1 (0.3 μM, plus NADPH and glutathione reductase). At the time points indicated, samples were withdrawn and assayed for sulfide by the methylene blue assay.

4. Discussion

4.1. Stimulation of FNR reconstitution by Grx in vitro

Here, the capacity of aerobic and anaerobic apoFNR as a receptor for a [4Fe–4S] cluster and a potential role for Grx or Trx in stimulating FeS cluster incorporation was studied. Presence of disulfides in apoFNR obviously inhibits reconstitution of [4Fe–4S] · FNR when glutathione is used as a reducing agent. Presence of wild-type as well as monothiol Grx significantly stimulates reconstitution. When glutathione is replaced by a more potent (artificial) reducing agent like dithiothreitol, this inhibition is no longer observed and Grx has no stimulatory effect. The stimulation was seen in particular in a reduction of the lag phase of aerobic apoFNR, suggesting that the increased disulfide content in aerobic apoFNR [13] is responsible for the lag phase in FNR reconstitution. The disulfide residues obviously are reduced more efficiently by the Grx than directly by glutathione. This is reminiscent of a similar effect during refolding of ribonuclease A by Grx1 and protein disulfide isomerase [32].

Overall, the results suggest that the Grx stimulate the reduction of cysteine disulfides in apoFNR which then in turn are capable of FeS cluster incorporation. According to this

assumption the cysteine disulfides are reduced during the lag phase, and in the second step the FeS cluster is inserted into apoFNR containing reduced cysteine residues. The latter reaction is only slightly stimulated by Grx. The reactions are not stimulated by Trx, whereas all isoforms of Grx show the stimulating effect.

4.2. Role for Grx in FeS cluster insertion in vivo?

Despite the stimulation of [4Fe–4S] · FNR reconstitution by the Grx in vitro, it is not clear whether the Grx have a stimulating effect on FeS cluster insertion in FNR or other FeS proteins in vivo, too. In particular under oxic conditions, it is feasible that the Grx have a role in the insertion of FeS clusters of proteins. Under oxic or microoxic conditions, O_2 diffuses rapidly into the cells [4,33] and could act as an oxidant for cysteine thiolates. Thus, in yeast mitochondria, the monothiol Grx5 (yGrx5) is required for formation of FeS proteins under aerobic conditions [30]. Grx5 is supposed to reduce disulfides of proteins under oxic conditions to enable FeS cluster insertion, very similar to the function suggested here for the Grx in FNR reconstitution. Thus, Grx might be of general significance for insertion of FeS clusters in proteins of *E. coli* under aerobic conditions. This situation would apply mainly to FeS proteins other than FNR which are synthesized and functional under aerobic conditions, similar to the situation with yGrx5 in yeast. For FNR, stimulation by Grx could be important under microoxic conditions when FNR is already active [4,33]. Under such conditions presence of cysteine disulfides in FNR could call for Grx for full stimulation of FNR (reconstitution). Stimulation of FeS cluster insertion could be significant for other proteins of aerobic metabolism carrying iron–sulfur clusters. A clear-cut answer to the role of Grx in the formation of iron–sulfur proteins will wait for in vivo studies with mutants lacking the complete Grx system which has, however, more general effects on aerobic growth of the bacteria.

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