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Desulfoferrodoxin of *Clostridium acetobutylicum* functions as a superoxide reductase

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Abstract Desulfoferrodoxin (*cac2450*) of *Clostridium acetobutylicum* was purified after overexpression in *E. coli*. In an *in vitro* assay the enzyme exhibited superoxide reductase activity with rubredoxin (*cac2778*) of *C. acetobutylicum* as the proximal electron donor. Rubredoxin was reduced by ferredoxin:NADP$^+$ reductase from spinach and NADPH. The superoxide anions, generated from dissolved oxygen using Xanthine and Xanthine oxidase, were reduced to hydrogen peroxide. Thus, we assume that desulfoferrodoxin is the key factor in the superoxide reductase dependent part of an alternative pathway for detoxification of reactive oxygen species in this obligate anaerobic bacterium.

Keywords: Oxidative stress; Superoxide reductase; Anaerobic bacteria; *Clostridium*

1. Introduction

Aerobic microorganisms defeat reactive oxygen species (ROS) such as superoxide anion and hydrogen peroxide via the catalase/superoxide dismutase (SOD) system. In anaerobes the function of this system is restricted or incomplete. Thus, in the genome of *Clostridium acetobutylicum* no catalase is annotated [1] and a function of the three annotated SODs could not yet been demonstrated. Furthermore, a disadvantage for anaerobes is also the production of oxygen by both enzymes. Nevertheless, *C. acetobutylicum* survives short periods of aeration [2]. Therefore, an alternative detoxification system must exist. Interestingly, all genes for a superoxide reductase (SOR) and peroxidase dependent detoxification pathway for reactive oxygen species proposed for *Pyrococcus furiosus* [3] are also present in *C. acetobutylicum* and other clostridia (Table 1). In *P. furiosus*, a SOR reduces superoxide to hydrogen peroxide with electrons from NAD(P)H involving rubredoxin (Rbo) and an oxidoreductase. Hydrogen peroxide is then further reduced to water by the peroxidase activity of rubrerythrin.

SORs are a class of non-heme iron enzymes and are subclassified into 1Fe-SORs and 2Fe-SORs. In the latter, the mononuclear iron active site [Fe(NHis)$_4$(SCys)] present in 1Fe-SORs is complemented by an additional N-terminal [Fe(SCys)$_4$] centre [4]. The function of the second iron site is not clear.

Desulfoferrodoxin (Dfx) which has been discovered in sulphate-reducing bacteria was shown to possess the ability to complement SOD deficient *Escherichia coli* strains [5,6]. This complementation was proposed to be a result of a NAD(P)H dependent SOR activity of Dfx. In turn, a desulfoferrodoxin knock out mutant of *Desulfovibrio vulgaris* showed increased oxygen sensitivity [7]. It has also been shown, that the two iron centre containing Dfx from *D. vulgaris*, *D. desulfuricans* and *Desulfoarculus baarsii* possess SOR activity *in vitro* [5,8,9]. In clostridia, enzymes with SOR activity in general or Dfxs in particular have not yet been analyzed. According to sequence data, *C. acetobutylicum* Dfx contains only one iron active site [Fe(NHis)$_4$(SCys)] and thus belongs to the 1Fe-SOR subclass [1].

In this study, the SOR activity of *C. acetobutylicum* Dfx representing a 1Fe-SOR was analyzed. Our *in vitro* results clearly demonstrate the SOR function of this enzyme. Thus, we assume that Dfx has an important role in the first part of a ROS-detoxification pathway in *C. acetobutylicum*.

2. Materials and methods

2.1. Reagents, proteins, and general procedures

Reagents and buffers were at least analytical grade. Xanthine oxidase, bovine liver catalase, ferredoxin:NADP$^+$ reductase (FNR), horseradish peroxidase (HRP), *Escherichia coli* superoxide dismutase (Fe-SOD) and *Clostridium pasteurianum* rubredoxin were purchased from Sigma. Concentrations were assumed to be those provided by Sigma. 10-Acetyl-3,7-dihydroxyphenoxazine (Amplex Red, AR) was from Synchem OHG. Reagents were used without further purification.

2.2. Cloning, expression and purification

Clostridium acetobutylicum ATCC824 genomic DNA was isolated according to Bertram and Dürre [10]. The rubredoxin- (*cac2778*) and desulfoferrodoxin genes (*cac2450*) were amplified using the following oligonucleotides: Rbo_Fw_BamHI 5′-CATTGGATCCATGGTATATCCCAATAAAAGG-3′, Rbo_Rev_PstI 5′-AATTTCTGCAGTTCTTCAGATGGCTCAAA-3′, Dfx_Fw_KpnI 5′-GGTAGGTACCATGAATAACGATTTATCAA-3′ and Dfx_Rev_BamHI 5′-GCTCAGGATCCTATATCTGCTTTCC-3′ introducing BamHI, PstI or KpnI restriction sites. The resulting fragments were cloned into a pASK-IBA 3 vector (http://www.IBA-GO.com). The resulting plasmids pI3*rbo* and pI3*dfx* were transformed into *E. coli* DH5α. The recombinant strains were grown in 500 ml Luria–Bertani broth supplemented with 100 μg ml^{-1} ampicillin at 37 °C under continuous shaking to an OD_{600} of 0.4–0.5. Gene expression was induced by addition of 0.2 μg ml^{-1} anhydrotetracycline. A better incorporation of iron into the proteins was achieved after addition of 40 μg ml^{-1} $FeSO_4 \cdot 7H_2O$ to the cultures. After 4 h cultures were harvested at $8000 \times g$ for 10 min and pellets were suspended in 5 ml buffer W (100 mM Tris–HCl, 150 mM NaCl) without EDTA. The disruption of the cells was achieved by ultrasonication using a MedLab Disintegrator Sonopuls HD60 apparatus for 1 min at a power of 30% and 50 pulses per

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Table 1
Genes of different Clostridia potentially involved in the SOR pathway

Organism	Rbo	Dfx	NROR	Normal Rbr	Reverse Rbr
C. acetobutylicum ATCC 824	*cac2778*	*cac2450*	*cac2448*	*cac2575* *cac3018*	*cac3597* *cac3598*
C. difficile Strain 630	*cd0828*	*cd0827*	*cd1623* *cd1157*	*cd2845*	*cd1524* *cd1474*
C. perfringens ATCC 13124	*cpe0777* *cpe0780*	*GI:18311480*	*cpf1269*	*cpe0135* *cpe1331* *cpe0855*	*cpe0689* *cpe0082* *cpe2618*
C. tetani E88	*ctc01387*	*ctc02454*	*ctc02521*	*ctc02638* *ctc01182*	*ctc00826*

min. The lysate was centrifuged for at least 30 min at 12000 × *g* and 4 °C. The resulting crude extract was loaded onto columns containing a Strep-Tactin-Sepharose-Matrix. Further purification steps were carried out as described in the IBA standard protocol (www.IBA-GO.com). The protein content of the elution fractions was determined using the Bradford assay [11]. Purity of the elution fractions was analyzed by SDS–PAGE [12].

2.3. Determination of SOR activity of Dfx in a cytochrome c assay

This method was based upon a superoxide dismutase assay [13]. The assay was performed in 1 ml cuvettes under aerobic conditions. All solutions contained a buffer of 100 mM Tris–HCl and 150 mM NaCl at pH 8.0. Cytochrome *c* (cyt *c*, 20 μM) was reduced in aerobic solutions resulting in an increasing absorbance at 550 nm. Generation of superoxide anions by addition of xanthine (0.2 mM) and xanthine oxidase (3.5 μg ml^{-1}) to the reaction mixture strongly enhanced this effect. SOD inhibits superoxide dependent reduction of cyt *c*. Enzymes (*E. coli* Fe-SOD or Dfx) were added after a constant baseline reaction was obtained. The tested Dfx enzyme concentrations were as follows: 0, 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, and 10 μM. SOD was taken as a control for the functionality of the assay. 1 U of SOD activity was defined as the amount of SOD causing 50% inhibition of reduction of cyt *c* under superoxide generation.

2.4. Reaction of Dfx in presence of Rbo, FNR and NADPH

SOR activity of Dfx was monitored essentially as described by Emerson [9]. A calibrated flux of superoxide generated by the xanthine/xanthine oxidase reaction as described for the standard SOD assay was used [13]. *C. acetobutylicum* Rbo was tested as the possible electron donor transferring the electrons to *C. acetobutylicum* Dfx. It was shown that NADH:rubredoxin oxidoreductase (NROR) can transfer electrons to Rbo and in turn regenerates the reduced form of this protein [14–16]. In this assay, the NROR was displaced by the NADPH dependent protein FNR from spinach. Heterologously expressed NROR is likely to be toxic for *E. coli* and could not be purified so far [14]. The reaction was monitored by measuring the NADPH consumption in a 1 ml cuvette as a decrease in absorbance at 340 nm (ε_{340} = 6220 M^{-1} cm^{-1}). All reagents and proteins were added from aerobic stock solutions at room temperature. The reaction mix contained 500 μM xanthine, 100 μM NADPH, 200 U ml^{-1} catalase, 1 μM FNR in a buffer of 50 mM sodium phosphate and 100 mM EDTA at pH 7.5. After 30 s *C. acetobutylicum* Rbo (1 μM) and after further 30 s either Dfx (1 μM) or Fe-SOD (250 U ml^{-1}) were added to this premix. When a constant baseline NADPH consumption rate was obtained 3.5 μg ml^{-1} of xanthine oxidase were added to start the reaction by superoxide generation.

2.5. Detection of H_2O_2

In presence of hydrogen peroxide, horseradish peroxidase (HRP) oxidizes AR to the fluorescent product resorufin. Assays were performed according to Seaver and Imlay [17] except that 100 μl of a 200 μM stock solution of AR in 50 mM potassium phosphate buffer pH 7.8 were added to 250 μl of the sample mix. The reaction mix contained 500 μM xanthine and 100 μM NADPH in a 50 μM MOPS buffer suspended with 0.1 mM EDTA at a pH of 7.8. H_2O_2 detection was started by addition of 10 μl of the HRP stock solution. This was followed by a subsequent addition of the missing reaction partners FNR, Rbo and Dfx at a concentration of 1 μM every 30 s. Xanthine oxidase (3.5 μg ml^{-1}) was added to start the SOR-reaction by superoxide generation. Production of resorufin was followed by an increasing absorbance at 560 nm.

2.6. Gel staining for SOD activity of Dfx

Non-denaturating 10% PAGE gels were prepared as described elsewhere [12] replacing ammonium persulphate by 28 μM riboflavin. Gels were then photopolymerized for 1 h under exposure to a 40 W lamp in a distance of 25 cm. Gel separation of the proteins was followed by determination of SOD activity according to Beauchamp and Fridovich [18]. After a shaking in 2.45 mM nitroblue tetrazolium for 20 min the gels were transferred to a solution containing 28 mM tetramethylenediamine, 36 mM potassium phosphate and 28 μm riboflavin at pH 7.8 for 15 min in complete darkness. The gels were washed with water and again illuminated until clear bands were visible.

3. Results

3.1. Purification of Dfx and Rbo from C. acetobutylicum

C. acetobutylicum Rbo (*cac2778*) and Dfx (*cac2450*) were expressed in *E. coli* DH5α labelled with a Strep-Tag and purified via Strep-Tactin column chromatography. This resulted in highly pure Rbo and Dfx protein fractions as shown exemplarily for Dfx in Fig. 1. Absorption spectra of the purified proteins are illustrated in Fig. 2. The spectrometric analysis of Dfx revealed a spectrum without any remarkable features between 400 and 700 nm (graph c). A peak with a maximum at 330 nm could be observed as it was also present in spectra of SOR from other bacteria [4]. The spectrum did not change under aerobic conditions for several hours indicating an extreme stability of the enzyme. In contrast, the spectrum of Rbo exhibited absorbance peaks at 350, 375, 492 and 565 nm (graph a). The peak at 492 nm is characteristic for an oxidized iron sulphur cluster of the [$Fe(SCys)_4$] type as it has been described for Rbos of other bacteria [19,20]. Thus, we assumed that in the purified Rbo of *C. acetobutylicum* this centre is occupied with iron and therefore this heterologously expressed protein should be functional. The purified Rbo was completely reduced by addition of an equimolar amount of sodium dithionite under anaerobic conditions (graph b). Reoxidation of Rbo (reappearance of the peak at 492 nm) occurred slowly within a few hours under aerobic conditions.

3.2. SOR activity of C. acetobutylicum Dfx

According to its proposed role as a SOR in ROS detoxification pathway *C. acetobutylicum* Dfx should be capable to act