Response of the Oxygen Sensor NreB to Air In Vivo: Fe-S-Containing NreB and Apo-NreB in Aerobically and Anaerobically Growing Staphylococcus carnosus

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The sensor kinase NreB from Staphylococcus carnosus contains an O2-sensitive [4Fe-4S]2+ cluster which is converted by O2 to a [2Fe-2S]2+ cluster, followed by complete degradation and formation of Fe-S-less apo-NreB. NreB · [2Fe-2S]2+ and apoNreB are devoid of kinase activity. NreB contains four Cys residues which ligate the Fe-S clusters. The accessibility of the Cys residues to alkylating agents was tested and used to differentiate Fe-S-containing and Fe-S-less NreB. In a two-step labeling procedure, accessible Cys residues in the native protein were first labeled by iodoacetate. In the second step, Cys residues not labeled in the first step were alkylated with the fluorescent monobromobimane (mBBr) after denaturing of the protein. In purified (aerobic) apoNreB, most (96%) of the Cys residues were alkylated in the first step, but in anaerobic (Fe-S-containing) NreB only a small portion (23%) were alkylated. In anaerobic bacteria, a very small portion of the Cys residues of NreB (9%) were accessible to alkylate in the native state, whereas most (89%) of the Cys residues from aerobic bacteria were accessible. The change in accessibility allowed determination of the half-time of 2 to 3 min. The [2Fe-2S]2+ cluster is further degraded, resulting in Fe-S-less or apoNreB. NreB is a permanent dimer in the active and inactive state. Disintegration of the [4Fe-4S]2+ cluster is coupled to the loss of the kinase activity of NreB, and NreB · [2Fe-2S]2+ and apoNreB are both inactive (20).

Typically, the in vivo function of regulators is assumed to correspond to that determined in vitro, although there are only a few examples where the functional state of a regulator has been studied in vivo. For NreB, the complex redox situation in the bacterial cell does not allow a direct transfer of the in vitro data and redox reactions to the cellular situation. Staphylococcus contains various reducing and oxidizing agents, including low-molecular-weight thiols (18, 22) and quinones. The compounds are able to react with Fe-S clusters and thiol groups of proteins and could affect the redox state or the reaction of NreB with oxygen. In addition, the presence of the two inactive forms, [2Fe-2S] · NreB and apoNreB, in vitro raises the question of the physiological form of inactive NreB in aerobic bacteria. Therefore, understanding the function of NreB in O2 sensing requires analysis of NreB in bacteria.

The state of NreB was studied in vivo by measuring the reactivity and accessibility of Cys residues to alkylating agents. NreB contains four Cys residues altogether, which are all ligands of the [4Fe-4S]2+ and [2Fe-2S]2+ clusters. The accessibility of the Cys residues should vary depending on the presence or absence of an Fe-S cluster and in this way allow differentiation of the Fe-S-containing forms from apoNreB. It turned out that only apoNreB is present in aerobically grown S. carnosus, whereas anaerobically grown bacteria carry essentially an Fe-S-containing form (NreB · [4Fe-4S]2+). In this...
way, the switch between aerobic and anaerobic NreB can be studied by measuring the accessibility of the Cys residues in vivo. The methods depend on a two-step labeling procedure for quantitative fluorescence labeling or on measuring the mass increase by mass spectrometry. Similar methods have been used to study the functional state of the oxygen sensor FNR of *Escherichia coli in vivo*. FNR binds a [4Fe-4S]°° cluster in the anaerobic state. [4Fe-4S]°° - FNR is converted to [2Fe-2S]°° - FNR and apoFNR after exposure to air (4, 9, 11, 17, 30, 31). ApoFNR was demonstrated as the physiologically relevant form in aerobically growing *E. coli* by labeling of the Cys residues in aerobically and anaerobically growing bacteria (26).

In this study, an advanced two-step labeling procedure was used to study quantitatively the accessibility of the Cys residues of NreB in vivo. In the first step, accessible residues were incubated with iodoacetate (IAA), which is highly accessible to surface-exposed Cys residues. After denaturation of the protein, the residual (Fe-S-protected) residues were incubated with the fluorescent reagent monobromobimane (mBBr). By this procedure, the accessible Cys residues of NreB were determined in vitro and in vivo under both aerobic and anaerobic conditions.

**MATERIALS AND METHODS**

**Growth of *S. carnosus*.** *S. carnosus* m1 (Δ*nreABC::ermB*) (6) carrying plasmid pCQE1nreB (Cam′) (34) for xylene-induced overexpression (yfl4 promoter) of *nreB* was grown at 37°C underoxic or anaerobic conditions in yeast extract production medium (YEPM) containing 45 g liter yeast extract (ServaBacte 24540), 50 mM sodium phosphate (pH 7.2), 40 mM glycerol, 10 mM sodium nitrate, and 10 mM ferric ammonium acetate. The cells were grown under aerobic conditions (300 ml in a 2-liter flask with baffles) on a rotary shaker (250 rpm) to an optical density at 578 nm (OD578) of 0.5, and then 150 mM xylene was added. Aerobic cells were harvested after 2 h. To obtain anaerobic cells, the aerobically grown bacteria were incubated after induction of NreB synthesis as described above for 5 h in sealed serum bottles under N2 (99.99%) at 37°C and were harvested subsequently.

**Isolation of NreB.** Aerobic NreB was prepared as NreB-His6 from *S. carnosus* strain m1(pCQE1nreB) after growth and induction under aerobic conditions (10). The protein was purified after induction under aerobic conditions as described previously (20), but without β-mercaptoethanol in the buffers. The procedure for purification of anaerobic NreB was the same as that for aerobic purification, but all steps were performed under anaerobic conditions in an anaerobic chamber (Coy), using anaerobic buffers (20) lacking β-mercaptoethanol. The isolation procedure included cell disruption for 4 cycles (4 s each) in an MP Fastprep24 system (0.1 mm beads; 5.0 m/s). The cell suspension was used to fill an air tight screw cup (2.0 ml microtube; Biologix), transferred outside the chamber, and broken by the use of Fastprep24. Afterwards, NreB-His6 was purified in the anaerobic chamber on a Ni-nitrilotriacetic acid (Ni-NTA) agarose column (Qiagen), yielding 5 to 10 mg of NreB protein in 5 ml of buffer.

**Labeling of NreB in vitro and in vivo by mBBr.** (i) Direct labeling. For labeling of NreB in vitro in aerobically growing bacteria, the bacterial growth was to an OD578 of 1.0 and induced by the addition of 150 mM xylene for synthesis of NreB. After 1 h, labeling was started by the addition of 0.2 μl of 0.8 M l-lysophastatin from a 0.5 U/ml stock solution (12), and 1 mM mBBr (Invitrogen) from a stock solution (10 mM in aceticamide [ACN]) was added (13, 14, 15, 26). During labeling, the bacterial culture was protected from light and shaken at 200 rpm under air for 10 min before the labeling reaction was stopped with 10 mM dithiothreitol (DTT), followed by sedimentation of the cells by centrifugation. When isolated NreB was incubated, maximal labeling by mBBr was achieved after a reaction time of 5 to 10 min. For anaerobic labeling, the bacteria were incubated after induction for 0.5 h in sealed serum bottles under an N2 gas phase (99.99%) and subsequently labeled as described for the aerobic sample, but in a glove box under N2, and using anoxic solutions throughout. The labeling was stopped after 10 min by adding 10 mM DTT. The labeled cells (aerobic or anaerobic) were collected by centrifugation and disrupted as described above for isolation of NreB. The cleared homogenate was subjected to immunoprecipitation (see below) and dissolved in sodium dodecyl sulfate (SDS) sample buffer (27). Three micrograms of purified protein, 100 μg of protein from cell homogenates, or 10 μg of precipitated protein was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (16). The proteins were blotted onto a polyvinylidene difluoride (PVDF) membrane (Millipore Immobilon-FL; pore size, 0.45 μm) as described previously (26) for further analysis.

(ii) Two-step or reverse labeling. In the first step, the native proteins were labeled with IAA (or where indicated) with N-ethylmaleimide (NEM). Labeling with IAA was performed by adding 1 mM IAA from a 500 mM stock solution in H2O, adjusted to pH 8 with NH3 (or by 1 mM NEM from a 1 M stock solution), to the sample (cell suspension or isolated NreB). After 15 min, the reaction was stopped by adding 1 mM DTT, and the sample was mixed with guanidine hydrochloride (2 M final concentration, from a 6 M stock). The Cys residues which became accessible after denaturing of the protein were then labeled with 2 mM mBBr as described above. For O2 inactivation kinetics, NreB was purified under anoxic conditions (20) in a glove box, using anoxic buffers (without β-mercaptoethanol). The anoxic protein was exposed to air by stirring 1 ml of the sample with a magnetic bar in a 15-ml Nalgene tube under air. After 0 min (before air exposure), 3 min, 6 min, 9 min, 12 min, and 15 min of exposure to air, samples were withdrawn and labeled with IAA as described above. After stopping the reaction with excess DTT, the protein was denatured with 2 M guanidine hydrochloride and the cysteine residues were labeled with mBBr as described for the two-step labeling procedure.

**Construction of NreB mutant strain IMW1884.** *S. carnosus* m1(pMW876). The C95S and C62S mutations were introduced into NreB by use of a QuikChange site-directed mutagenesis kit (Stratagene) and shuttle plasmid pCQE1nreB (Cam′) (34). The plasmid (pCQE1nreB,NreC operon) was transformed into *E. coli* DH10B, and the resulting m4-mer-fNreB-cmB operon. The mutations were introduced using primers F-C95S+C62S(TCG)-NreB (GAC GAA TGC AGT TTC TCG CAG ATC GAG AGA ATTA T) and R-C95S+C62S(TCG)-NreB (AAAT CTT CCG ATC TGG AAA CTG CAC ATG TTG CTC). The NreB mutant encoding NreB(C95S C62S) on the resulting plasmid, pBR734nreB*C, was amplified using primers p1 (5’ GTT GTC TCC TAA GAT CTA TCG ATT GGT 3’) and p2 (5’ CCG TTA GGG AGT TAA CCA GAT G 3’) and cloned into the BglII and KpnI sites of the staphylococcal expression plasmid pCQE1 (6), yielding plasmid pMW876. *S. carnosus* m1 was transformed with the plasmid. Induction by xylose and overproduction and isolation of NreB(C95S C62S) was conducted as described for wild-type NreB.

**Quantification of labeling.** After SDS-PAGE of the proteins and blotting to a PVDF membrane, mBBr fluorescence and NreB immunostaining were determined. The fluorescence on the PVDF membrane derived from mBBr was recorded as described for wild-type NreB.

**Acidification of shuttle plasmid.** For mass spectrometry, purified native NreB in buffer (50 mM sodium phosphate, 300 mM NaCl, 150 mM imidazole at pH 7.2) was labeled with 5 mM IAA (from a 500 mM stock solution in H2O, adjusted to pH 8 with NH3), 10 mM mBBr, and 0.45 mM NEM for 1 h. The resulting labeled NreB was purified from the reaction mixture by gel filtration on a Sephadex G-25 column. The labeled NreB was used for mass spectrometry. For mass spectrometry, purified native NreB in buffer (50 mM sodium phosphate, 300 mM NaCl, 150 mM imidazole at pH 7.2) was labeled with 5 mM IAA (from a 500 mM stock solution in H2O, adjusted to pH 8 with NH3), 10 mM mBBr, and 0.45 mM NEM for 1 h. The resulting labeled NreB was purified from the reaction mixture by gel filtration on a Sephadex G-25 column. The labeled NreB was used for mass spectrometry. For mass spectrometry, purified native NreB in buffer (50 mM sodium phosphate, 300 mM NaCl, 150 mM imidazole at pH 7.2) was labeled with 5 mM IAA (from a 500 mM stock solution in H2O, adjusted to pH 8 with NH3), 10 mM mBBr, and 0.45 mM NEM for 1 h. The resulting labeled NreB was purified from the reaction mixture by gel filtration on a Sephadex G-25 column. The labeled NreB was used for mass spectrometry.
sequencing grade; both from Roche Diagnostics). All buffers were prepared without glycerol. Digestion was controlled by running a sample on the SDS-PAGE gel. The digested protein as well as labeled intact protein from aerobic and anaerobic bacteria was frozen in liquid N$_2$ and analyzed by mass spectrometry. The fragments were checked for the presence of the Cys-containing peptides by screening for peptides with masses corresponding to the requested peptide and the mass increases due to incorporation of the acetyl label (58 Da per Cys residue) for the Asp-N and Glu-C peptides.

Matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry. Sinapic acid (Sigma Aldrich, Taufkirchen, Germany) and α-cyano-4-hydroxy-cinnamic acid (Bruker Daltonics, Bremen, Germany) were used as matrices. External calibration of the spectra was performed using peptide calibration standard II (Bruker Daltonics, Bremen, Germany). The samples were desalted with ZipTip μC$_18$ tips (Millipore, Billerica, MA), using ACN (BioSolve, Westford, MA), trifluoroacetic acid (TFA; Mallinckrodt Baker, Phillipsburg, NJ), and MilliQ water (prepared by a Millipore water purification system (Billerica, MA)).

Sample preparation for MALDI-TOF. To obtain better signal-to-noise levels, the samples were desalted with a pipette tip (ZipTip μC$_18$; Millipore) and adjusted to 1.5 μl according to the following procedure. First, the ZipTip was washed twice with 50% aqueous ACN solution, and then it was washed two times with 0.1% TFA. Afterwards, 1.5 μl of sample was picked up, rinsed back and forth in the pipette tip, and pushed out. This procedure was repeated 10 times, using a total volume of 15 μl of sample. After being washed with 0.1% TFA and MilliQ water three times, the samples were eluted on the target with the matrix. Sinapic acid was used (10 mg/ml solved in 2/1 ACN/0.1% TFA [vol/vol]) for all samples digested with endoproteinase Glu-C (Roche Diagnostics). The spots were allowed to dry at room temperature.

All MALDI-TOF mass spectra were acquired on a Bruker Reflex III mass spectrometer (Bruker Daltonics, Bremen, Germany). An MTP AnchorChip 600/384 (Bruker Daltonics, Bremen, Germany) was used as the target. The MALDI-TOF mass spectrometer utilizes a pulsed nitrogen laser, emitting at 337 nm. The TOF mass spectrometer utilizes a pulsed nitrogen laser, emitting at 337 nm. The 384 (Bruker Daltonics, Bremen, Germany) was used as the target. The MALDI-TOF mass spectrometer utilizes a pulsed nitrogen laser, emitting at 337 nm. The laser strength was kept at about 35% to obtain an optimum signal-to-noise ratio. To enhance the spectral resolution, all spectra were averaged for each mass spectrum. The laser strength was kept at about 35% to obtain an optimum signal-to-noise ratio. To enhance the spectral resolution, all spectra were averaged for each mass spectrum.

RESULTS

Direct (one-step) labeling of Cys residues of NreB from aerobically and anaerobically growing S. carnosus. NreB contains four cysteine residues altogether, which are required for the binding of the Fe-S cluster (20). Labeling of accessible Cys residues was used as an approach for differentiating Fe-S-containing and Fe-S-free forms of NreB in vivo and in vitro and to define the actual state of NreB in aerobically and anaerobically grown cells. For labeling, the fluorescent reagent mBBr, which reacts specifically with Cys thiols, was used. mBBr and IAA are sufficiently membrane permeant in the minute range for labeling without cell disruption (19, 28). mBBr becomes fluorescent after reaction with thiols. Labeling of the Cys residues of NreB was studied in situ in growing S. carnosus proteins without prior permeabilization of the cytoplasmic membrane to avoid perturbation of the bacteria (26). The labeling was performed after partial digestion of the cell wall by lyso- staphin to allow accession of the reagent to the membrane. After labeling and stop of the reaction by addition of DTT, the bacteria were broken and NreB was sedimented by NreB-specific antisera and protein A Sepharose. The proteins of the sediment were subjected to SDS-PAGE, and after blotting of the proteins to a PVDF membrane, the fluorescence of protein bands was visualized (Fig. 1). One or two proteins in the M$_r$ range of NreB were fluorescent due to incorporation of mBBr. The fluorescent bands at the expected position for NreB showed high intensities after induction of NreB production and were lacking in noninduced cells, suggesting that the bands were derived from NreB. The fluorescence was much higher for aerobically than for anaerobically grown bacteria. In the immuno blot, the protein bands corresponding to the fluorescent bands reacted heavily with anti-NreB serum, and the reaction intensities for NreB were fluorescent due to incorporation of mBBr. The fluorescence of protein bands was visualized (Fig. 1). One or two proteins in the M$_r$ range of NreB were fluorescent due to incorporation of mBBr. The fluorescent bands at the expected position for NreB showed high intensities after induction of NreB production and were lacking in noninduced cells, suggesting that the bands were derived from NreB. The fluorescence was much higher for aerobically than for anaerobically grown bacteria. In the immuno blot, the protein bands corresponding to the fluorescent bands reacted heavily with anti-NreB serum, and the reaction intensities for NreB were fluorescent due to incorporation of mBBr. 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formed with the NreB(C59S C62S) mutant that retained only two Cys residues, the level of labeling increased only to levels corresponding to about 50% of the level of wild-type NreB. The labeling remained at this level even in the presence of a large excess of mBBr. The responses of the wild type and the Cys mutant demonstrate that the amount of mBBr incorporated is related to the number of accessible Cys residues. By using fully labeled NreB and NreB(C62S C59A) as references, labeling of other NreB samples can be evaluated quantitatively and used to determine the number of accessible Cys residues. Labeling of native NreB by mBBr without the addition of guanidinium hydrochloride (direct labeling), on the other hand, gave lower yields in labeling and was not used for quantitative labeling studies.

Quantitation of accessible Cys residues of aerobic and anaerobic NreB (in vivo and in vitro). For quantitative evaluation of the number of accessible Cys residues, a two-step labeling procedure was used which included a denaturing step. By this procedure, the lysostaphin-treated bacteria were first incubated with iodoacetate to label accessible Cys residues. Iodoacetate is able to diffuse across the cytoplasmic membrane into the cytoplasm at a sufficient rate for the labeling procedure (1, 25). After stoppage of the labeling reaction by the addition of DTT, the bacteria and proteins were denatured by the addition of 2 M guanidinium hydrochloride. Subsequently, all Cys residues that had not been labeled in the first reaction with IAA were labeled with mBBr (step two). After separation of the proteins by SDS-PAGE, proteins corresponding to the mass of NreB showed a fluorescent band (Fig. 3A). The band increased in intensity in the bacteria induced for NreB production. The same band from anaerobically grown bacteria showed a much higher intensity in fluorescence than that from aerobically grown bacteria. The amounts of NreB, however, were comparable for aerobically and anaerobically grown bacteria (Fig. 3B), suggesting that the specific fluorescence was increased in the anaerobic bacteria.

The difference in labeling cannot be explained by simple changes in the redox state of the Cys residues in NreB, since high levels of labeling for the native protein (i.e., labeling by IAA) were found for aerobically grown bacteria and low levels of NreB were found for anaerobically grown bacteria. The labeling can be explained, however, by assuming protection of the Cys residues from labeling by bound Fe-S clusters in anaerobic bacteria. Moreover, the finding is in agreement with studies on bound Fe-S clusters and accessible Cys residues. Therefore, two-step labeling of the Cys residues of NreB can be used to differentiate Fe-S-containing NreB from apoNreB.

In vivo and in vitro state of NreB by reverse labeling of Cys residues. For quantitative analysis of the accessibility of the Cys residues, generally the two-step labeling procedure was used. In the experiment shown in Fig. 4, the accessibility of Cys residues was compared for NreB under aerobic and anaerobic conditions in vivo and in vitro. The Cys residues accessible in the native and denatured proteins were labeled differentially with IAA and mBBr. The mBBr fluorescence therefore represents the Cys residues that became accessible only after denaturing of the protein, i.e., the Fe-S-protected residues. The difference in the mBBr label compared to the maximal label, on the other hand, represents the Cys residues that were labeled already by IAA in the native protein.

In aerobically grown bacteria, only a small portion (9%) of the Cys residues were labeled by mBBr, since most of the residues were labeled by IAA in labeling step 1. In the anaerobic bacteria, most of the Cys residues (89%) were accessible to labeling by mBBr after denaturing of the protein. When in a control experiment the proteins of the bacteria were denatured by guanidinium hydrochloride before the first labeling step, the second reaction only a small portion was labeled by mBBr for both types of bacteria. Therefore, the differences in labeling can be explained by different accessibilities, whereas other factors apparently play no role. Overall, the Cys residues in NreB proteins from aerobic and anaerobic bacteria are largely different in their accessibility to labeling reagents. The
corresponded to that described for panel A and for Fig. 3.

Before the first labeling step, in the second labeling reaction tured in a control experiment by guanidinium hydrochloride NreB had lost the Fe-S cluster. Again, when NreB was dena-

measurable part of the anaerobic NreB is no longer in the

sponded to the Fe-S-protected residues, it was assumed that a

compared to 89%). Since the mBBr-reacting residues corre-

proteins. This was similar to the case of the protein in bacteria,

only in the second step with mBBr, that is, after denaturing the

protein. This was similar to the case of the protein in bacteria,

most of the Cys residues (77%) became accessible to labeling

in vivo

in vitro

O2 N2 O2 N2 O2 N2 O2 N2 O2 N2

IAA IAA IAA IAA IAA

GnHCl GnHCl GnHCl GnHCl GnHCl

mBBr mBBr mBBr mBBr mBBr

1 2 3

FIG. 4. Labeling of Cys residues in NreB by two-step labeling with IAA (native labeling) and mBBr (labeling of denatured protein). Label-
ing of NreB in growing bacteria (A) and of isolated NreB (B) is shown. (A) Anaerobically (N2) and aerobically (O2) growing (OD578 =

1.0) S. carnosus ml(pCQE1nreB) cells were incubated with iodoace-
tate (1 mM, 10 min) after finishing nreB induction (at an OD760 of 0.5;

induction for 2 h with 150 mM xylose). After the addition of DTT (1

mM), 2 M guanidinium hydrochloride and 2 mM mBBr were added (experiment 1). In experiment 2, labeling by iodoacetate was per-
formed as in experiment 1, but the guanidinium hydrochloride was

added before labeling by IAA. In experiment 3, the bacteria were

incubated with 2 M guanidinium hydrochloride (without iodoacetate

labeling) and then labeled by 1 mM mBBr. Protein (50 μg per lane)

dissolved in SDS sample buffer and subjected to SDS-PAGE. After

blotting of proteins to a PVDF membrane, the mBBr fluores-

cence and the amount of NreB were determined as described in the

legends to Fig. 2 and 3. Each bar gives the labeling by mBBr (white

part of the bar) and by IAA (gray part of the bar). Labeling by IAA was

calculated as the difference in the mBBr label compared to the maxi-

mal label (mBBr label in experiment 3). (B) The same labeling

procedure as for panel A was performed with aerobically (O2) or

anaerobically (N2) prepared NreB (20). The labeling procedure corre-

sponded to that described for panel A and for Fig. 3.

difference is observed only when the proteins are in the native

during the labeling in step 1, demonstrating that the differences represent the state of the native protein.

When the two-step labeling experiment was performed in vitro (Fig. 4B) with isolated NreB, the labeling pattern was similar to that in bacteria. The labeling of aerobic NreB was

very high in the first step, i.e., labeling of the native protein by

IAA, and low in the second step (labeling by mBBr after denaturing the protein). In the anaerobic [4Fe-4S]2+ · NreB, most of the Cys residues (77%) became accessible to labeling only in the second step with mBBr, that is, after denaturing the protein. This was similar to the case of the protein in bacteria, but the amount of mBBr-labeled residues was smaller (77% compared to 89%). Since the mBBr-reacting residues corre-

sponded to the Fe-S-protected residues, it was assumed that a measurable part of the anaerobic NreB is no longer in the

[4Fe-4S]2+ · NreB form. In agreement with this observation, it was suggested earlier (20) that part of the isolated anaerobic

NreB had lost the Fe-S cluster. Again, when NreB was dena-

tured in a control experiment by guanidinium hydrochloride before the first labeling step, in the second labeling reaction only a small portion was labeled by mBBr. This applies in the same way to aerobic and anaerobic NreB.

Overall, it appears that labeling by iodoacetate and mBBr in the two-step procedure detects the number of accessible Cys residues in vivo and in vitro in a very similar way and that the labeling method allows differentiation of Cys residues that bind an Fe-S cluster and those not involved in binding. Accordingly, the differences in Cys residues that are accessible in the native state under aerobic and anaerobic conditions represent Fe-S-protected Cys residues. Iodoacetate obviously does not dis-

place the Fe-S cluster, which is demonstrated by the high level of protected Cys residues under anaerobic conditions.

Numbering of Cys residues accessible to iodoacetate by mass spectrometry. Aerobically or anaerobically prepared NreB was incubated in the native form with an excess of iodoacetate in order to label accessible Cys residues. Prior to being labeled, the sample was incubated with small amounts of DTT in order to keep the available Cys residues in the reduced state. After labeling and subsequent inactivation of unreacted iodoacetate by an excess of DTT, the samples were digested with protease Glu-C, which cleaves specifically at the C terminus of Cys residues. The peptide mixture was then analyzed by mass spectrometry, with particular attention to the presence of the Cys-containing peptides (Fig. 5). Protease Glu-C generates principally three Cys-containing peptides from NreB (PepC1

![Mass spectrometry of Glu-C (A) and Asp-N (B)-derived peptides of NreB from aerobically (O2) or anaerobically (N2) grown bacteria after direct labeling by iodoacetate.](http://jb.asm.org/)
peptides are labeled with the position of the corresponding Glu residue in NreB. Form, PepC3-Ac2, with two acetyl residues (2,457 Da), was found only as the alkylated forms (PepC1-Ac, PepC2-Ac, and PepC3-Ac2), whereas the unmodified forms were absent. This is shown in Fig. 5A for PepC3, where the fully alkylated masses corresponding to PepC1, PepC2, and PepC3 in the unmodified form and to peptides with masses corresponding to PepC1, PepC2, and PepC3 (Table 1). One of the peptides (PepC3) contains two Cys residues. The digest was screened for peptides with masses corresponding to PepC1, PepC2, and PepC3 after alkylation of the Cys residues with iodoacetate and digestion with Glu-C and Asp-N show that all of the Cys residues in aerobic NreB are accessible to alkylation. From anaerobic NreB, on the other hand, PepC3 was present as the unmodified form only (2,336 Da), and none of the alkylated forms of PepC3 was detected. PepC1 and PepC2, which were also expected in the nonmodified form, were not detected, suggesting that it is difficult to identify the nonalkylated forms of the latter peptides. The absence of the alkylated forms of PepC1 and of PepC2 (which were detected in the aerobic forms of NreB) suggests that the corresponding forms are not present in anaerobic NreB. In a preparation of aerobic NreB that was alkylated with iodoacetate and then digested by Asp-N, the peptide PepN1 (2,389 Da) was identified, which might represent the peptide containing Cys59 and Cys62 (Fig. 5B). The Asp-N digest of aerobic NreB contained high levels of PepC3, with two acyl residues (2,457 Da), whereas the unmodified (or protected) form and the form with one acyl residue (2,389 Da and 2,447 Da, respectively) were present at much lower levels. Therefore, the combined data from labeling with iodoacetate and digestion with Glu-C and Asp-N show that Cys residues of aerobically incubated NreB are accessible to alkylation, whereas those from anaerobically incubated Fe-S-containing NreB are protected from alkylation by iodoacetate and incubated with mBBr. Earlier, the kinetics for the conversion of NreB to apoNreB conversion to apoNreB by O2. Mo¨ssbauer studies have shown that anaerobic NreB was high before starting air exposure (about 80 to 90% of the total Cys residues), which is characteristic for the presence of NreB-[4Fe-4S]2+. The number of mBBr-accessible residues decreased exponentially with increasing time of air exposure. After 15 min, only 11% of the Cys residues were still accessible to labeling by mBBr, demonstrating that most of the NreB was converted to apoNreB. The labeling by iodoacetate was calculated as the difference in mBBr-accessible to total accessible Cys residues (Fig. 6). The half-time for the change in accessibility was about 6 min, and after 15 min, approximately 90% of the total Cys residues became accessible.

### DISCUSSION

#### Use of Cys labeling for functional studies of NreB in vivo and in vitro

Møssbauer studies have shown that anaerobic NreB present in the digest from aerobic NreB and absent in those from anaerobic NreB.

**Kinetics of [4Fe-4S]2+ · NreB conversion to apoNreB by O2.**

Earlier, the kinetics for the conversion of NreB · [4Fe-4S]2+ to apoNreB conversion to apoNreB by O2. Mo¨ssbauer studies have shown that anaerobic NreB was high before starting air exposure (about 80 to 90% of the total Cys residues), which is characteristic for the presence of NreB · [4Fe-4S]2+. The number of mBBr-accessible residues decreased exponentially with increasing time of air exposure. After 15 min, only 11% of the Cys residues were still accessible to labeling by mBBr, demonstrating that most of the NreB was converted to apoNreB. The labeling by iodoacetate was calculated as the difference in mBBr-accessible to total accessible Cys residues (Fig. 6). The half-time for the change in accessibility was about 6 min, and after 15 min, approximately 90% of the total Cys residues became accessible.

#### FIG. 6. Kinetics of conversion of purified anaerobic NreB · [4Fe-4S]2+ to apoNreB by exposure to air.

Isolated anaerobic NreB · [4Fe-4S]2+ (1 mg/ml) was exposed to air and mixed at the indicated time points with iodoacetate. After stoppage of the iodoacetate reaction with DTT, the sample was denatured by 2 M guanidinium hydrochloride and incubated with mBBr. After SDS-PAGE and immunoblotting, the specific label of mBBr fluorescence (■) was determined. The Cys content in a sample which was labeled only with mBBr was set to 100%. The amount of label (or labeling of Cys residues) by iodoacetate (▲) was calculated as the difference between the 100% label and the mBBr label for the given sample.
NreB \cdot [4Fe-4S]^2^+ protein is converted in vitro in the presence of O_2 to NreB \cdot [2Fe-2S]^2^+ and, further to apoNreB (20). On the basis of these results, the Cys labeling studies demonstrate that the same reactions take place in vivo in the cells of S. carnosus and that apoNreB is the physiological form in aerobically growing bacteria. Overall, oxygen causes similar reactions and conversions of NreB in vitro and in vivo, and NreB \cdot [4Fe-4S]^2^+ and apoNreB are the physiologically relevant forms under aerobic and anaerobic conditions, respectively (Fig. 7). The experiments also show that apoNreB is formed in vitro from [4Fe-4S]^2^+ \cdot NreB, with a half-time of 6 min, which fits the kinetics of [4Fe-4S]^2^+/[2Fe-2S]^2^+ cluster conversion, with a half-time of about 2.5 min, that was obtained by Mössbauer studies.

Quantitative evaluation of the accessibility of the Cys residues of NreB and the clear-cut differentiation between apoNreB and Fe-S-containing NreB required a two-step labeling procedure. In this procedure, the first labeling step is performed with the native protein, using IAA, and the second labeling step is performed with the denatured protein, using mBBr. The quantitative labeling by IAA was confirmed by alkylation and mass spectrometry. The two-step procedure using IAA and mBBr allowed work in vitro and in situ in growing bacteria when the NreB concentrations were relatively low.

Mössbauer studies have been used to differentiate [4Fe-4S]^2^+ and [2Fe-2S]^2^+-containing forms of FNR in vivo (24). NreB is not overproduced to sufficient levels to allow Mössbauer studies with cells of S. carnosus. Cys labeling therefore enables studies on proteins in vivo which cannot be overproduced to extents required for Mössbauer studies. More importantly, the studies can be performed with moderate levels of NreB overproduction, and physiological conditions can be applied. According to an estimation from immunoblotting, wild-type S. carnosus contains 0.16 \mu g of NreB/mg of total cell protein or less (corresponding to an approximate concentration of 0.4 \mu M NreB in the bacterium). In the expression strain, about 4 \mu g of NreB/mg of total cell protein was found under the conditions of the labeling experiments. This suggests an about 30-fold increase compared to the wild type, which is, however, still a factor of 10 below the levels required for in vivo Mössbauer spectroscopy (24). Even more importantly, the labeling experiments can be performed at low cell densities and under aerobic and anaerobic conditions, whereas for in vivo Mössbauer spectroscopy, very high cell densities are required, which makes maintenance of aerobic conditions and studies on the aerobic-anaerobic switch very difficult. Therefore, labeling of the Cys residues of labile Fe-S clusters is a useful method for studies on cluster degradation in vivo and in vitro and complements Mössbauer studies.

**Functional state of NreB in situ (in vivo).** Oxygen sensing is generally complicated by the fact that the primary stimulus is often not well defined. Other oxidants might have similar effects and are interchangeable with molecular oxygen. Therefore, it is important to study oxygen sensors in vivo in bacteria and to verify the in vitro reactions. This approach is of particular significance for NreB, for which two different air-inactivated forms ([2Fe-2S]^2^+ \cdot NreB and apoNreB) have been shown in vitro. Anaerobically growing S. carnosus contained only NreB with bound Fe-S clusters; there were no significant levels of apoNreB. Since [4Fe-4S]^2^+ \cdot NreB represents the only active form of NreB and was isolated as the major form from anaerobic bacteria (20), the Fe-S-containing form of NreB in the aerobic bacteria has to be [4Fe-4S]^2^+ \cdot NreB. The aerobically growing bacteria, on the other hand, contained only apoNreB; [2Fe-2S]^2^+ \cdot NreB was not present in significant amounts. Thus, apoNreB is the physiologically relevant form in aerobic S. carnosus. Many Gram-positive bacteria, such as *Staphylococcus* and *Bacillus*, lack glutathione, which is replaced by cysteine and other low-molecular-weight thiols (18, 22), Glutathione and cysteine equilibrate only slowly with molecular oxygen and are present in the cytoplasm essentially in the reduced state, even under aerobic conditions, when O_2 diffuses into the cytoplasm.

It is not clear whether NreB \cdot [2Fe-2S]^2^+ is also of physiological significance to this bacterium. This form of NreB might be important for transitional stages when the oxygen supply is limited in time or concentration. The presence of NreB \cdot [2Fe-2S]^2^+ as an intermediate might facilitate and accelerate switching between the active and inactive forms of the sensor kinase. It is, however, also conceivable that the [2Fe-2S]^2^+ form is a degradation intermediate which is formed only for chemical reasons, without physiological significance. Similarly, the role of the [2Fe-2S]^2^+ cluster of FNR is not clear, which could indicate that [2Fe-2S]^2^+ intermediates are generally formed during degradation of O_2-labile [4Fe-4S]^2^+ clusters.

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*Fig. 7. Scheme for the function of the NreBC two-component system as an O2 sensor. For NreB, the kinase domain with the conserved His (H) residue and the PAS domain with the conserved Cys residues (C) are shown. The figure summarizes the reactions at the sensor kinase NreB and the Fe-S cluster during transition from anaerobic [4Fe-4S]^2^+ \cdot NreB to the air-inactivated forms. NreB is a permanent dimer (20), but only one monomer of the protein is shown. The final and physiologically relevant air-inactivated form is apoNreB in vivo and in vitro. [2Fe-2S]^2^+ \cdot NreB is formed in vitro as an intermediate, but the presence of this form has not been tested in vivo. The overall process of [4Fe-4S]^2^+ \cdot NreB → apoNreB conversion has a half-time of about 6 min, and the half-time of [4Fe-4S]^2^+ \cdot NreB → [2Fe-2S]^2^+ \cdot NreB conversion is about 2.5 min. The function of [2Fe-2S]^2^+ \cdot NreB in vivo is not known. The anaerobic [4Fe-4S]^2^+ \cdot NreB protein has high kinase activity, resulting in the phosphorylation of NreB and NreC. NreC-P activates transcription of the target genes narGHJI, narT, and nirBD.*
REFERENCES


