Characterization of bacterial cytochrome cd_{1}-nitrite reductase as one enzyme responsible for catalysis of nitrosation of secondary amines

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Bacterial formation of carcinogenic N-nitroso compounds may play a role in the etiology of human cancer. Biochemical and immunological studies in denitrifying bacteria (Pseudomonas aeruginosa) strongly support the identification of cytochrome cd_{1}-nitrite reductase as the enzyme responsible for the catalysis of nitrosation through the production of nitric oxide or NO^{+}-like species. Interestingly, electron paramagnetic resonance studies have shown that large quantities of nitric oxide or NO^{+}-like species were also produced by non-denitrifying enterobacteria (Escherichia coli, Proteus morganii).

Introduction
Chronic bacterial infections are recognized or suspected risk factors associated with several malignancies, including cancer of the stomach, urinary bladder and uterine cervix. Bacterial formation of carcinogenic N-nitroso compounds (NOC*) in situ is one possible mechanism that may play an etiological role in these cancers. A number of studies have now unequivocally shown that bacterially mediated formation of nitrosamines can occur in vitro and in vivo, both in animal models and in human cancer cases.

We have succeeded in purifying the nitrosating enzyme from two denitrifying organisms, Pseudomonas aeruginosa and Neisseria mucosae. We observed a strong nitrite-reductase activity in a chromatographic fraction containing this nitrosating protein. We also observed a strong nitrite-reductase activity in a chromatographic fraction containing this nitrosating protein. In addition, a mutant strain of Pseudomonas stutzeri, which lacks the cd_{1}-nitrite reductase (cd_{1}-NIR), was devoid of any capacity to nitrate secondary amines. We therefore performed several experiments, as described in this paper, to ascertain whether cd_{1}-NIR is one of the bacterial enzymes responsible for catalyzing the formation of carcinogenic NOC via the production of nitric oxide (NO) or NO^{+}-like species.

Material and methods
Chemicals
Sodium nitrite, sodium nitrate, morpholine hydrochloride (MOR) and N-methyl-pentylamine were obtained from Merck; N-nitrosomorpholine (NMOR), methylviologen, d,l-dithiothreitol, 3,3',5,5'-tetramethylbenzidine, 3,3'-diaminobenzidine, anti-rabbit IgG-peroxidase conjugate, DMSO, BSA and hydrogen peroxide from Sigma; and 3,3'-diaminobenzidine, anti-rabbit IgG-peroxidase conjugate, DMSO, BSA and hydrogen peroxide from Sigma; and diethyldithiocarbamic acid ethyl ester (DETC) from Eastman-Kodak. All other chemicals were of analytical grade and obtained from either Merck or Sigma. Nitrosamines, as carcinogens, have to be handled under appropriate safety conditions.

Bacterial cultures
Bacteria were grown in a minimal medium (five times diluted hemocult broth, Institut Pasteur, Lyon, France) under anaerobiosis in the presence of 5 mM NaNO_{2} or 10 mM NaNO_{3}. After washing with 0.9% (w/v) saline, the bacteria were resuspended in 0.9% saline. Protein was determined by the Lowry method, using BSA fraction V (Sigma) as a standard. When DETC was present in the sample, protein concentration was determined with Coomassie blue.

Isolation of the nitrosating enzyme
Isolation and purification of the nitrosating enzyme were carried out at 4°C as previously described in detail. The cd_{1}-NIR was purified from Pseudomonas aeruginosa according to the method described by Parr et al.

Nitrosation assay
Nitrosation activity was measured as previously described. Briefly, a reaction mixture containing 0.1 M Tris-Cl buffer, pH 7.2, 25 mM MOR, 25 mM NaNO_{2}, 20 mM DETC and the bacterial suspension or the enzymatic fraction (final volume 0.5 ml) was incubated at 37°C for 1 h. Control experiments were carried out with 0.9% saline, instead of enzymatic fraction or bacterial suspension. NMOR was analyzed with a gas chromatograph interfaced to a thermal energy analyser model 502 (Thermoelectron Corporation, Waltham, MA). Specific nitrosation activity was defined as nmol or mmol NMOR formed/mg protein/h, after subtraction of the control value obtained in a cell- or enzyme-free assay.

Non-competitive ELISA for nitrosating enzyme and cd_{1}-NIR
Polyclonal antibody against the cd_{1}-NIR of Pseudomonas aeruginosa was kindly provided by Dr M. Coyne (Michigan State University, USA). Falcon 96 well microtest plates were coated with various concentrations of nitrosating enzyme or cd_{1}-NIR in 50 μl of PBS and incubated at 4°C overnight. The remaining protein-binding sites were blocked by incubation with 100 μl/well of 0.2% BSA for 30 min at 37°C. The plates were then washed twice with the same solution. Following the addition of 100 μl of an appropriately diluted serum per well, the plates were incubated for 60 min at 37°C. The plates were again washed, and 50 μl of anti-rabbit IgG-peroxidase conjugate (1:5000 dilution in PBS) were added and incubated for 60 min at 37°C. After the plates were washed, 50 μl of a substrate solution containing 1 mg 3,3',5,5'-tetramethylbenzidine in 0.1 ml DMSO and 2 μl of H_{2}O_{2} (30% w/w) in 10 ml citrate buffer, pH 5.0, were added and incubated for 15 min at room temperature. The reaction was stopped with 50 μl 1 M HCl and the absorbance was read at 450 nm with an EL 312 plate reader (Bio-Tek Instruments).

Electron paramagnetic resonance (EPR) spectroscopy
One hundred micromolar enzyme or 10^{11} bacteria/ml, 25 mM NaNO_{2} and 10 mM DETC were allowed to react at room temperature for 30 min with or without the addition of 25 mM MOR. Samples were transferred into EPR tubes and were rapidly frozen in liquid nitrogen and stored at 77 K until needed. EPR spectra were recorded at 77 K using a Varian E 109 spectrometer, under the following experimental conditions: microwave power 10 mW, microwave frequency 9.18 GHz, modulation amplitude 1 mT, time constant 0.5 s, 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was used as a field marker with g = 2.0036.

Peptide analysis
Fifty micrograms of the nitrosating enzyme and 50 μg of cd_{1}-NIR were treated with 2.5 mg cyanogen bromide in 70% formic for 16 h at 25°C. A control was performed with 70% formic for each enzyme. After drying under nitrogen, samples were analyzed by SDS-PAGE on 15% acrylamide gels and stained with Coomassie blue.

Results
The experiments described below were carried out in order to ascertain whether cd_{1}-NIR is the enzyme responsible for catalysis of nitrosation in denitrifying bacteria.
Nitrosating activity of Pseudomonas nirS::tn5

The mutant strain MK202 of Pseudomonas stutzeri ZoBell ATCC 14405 is known to have a tn5 insertion in the nirS gene, the structural gene for cd1-NIR, and has no nitrite-reductase activity (12).

According to our nitrosation assay, this mutant lacked the capacity to catalyze the nitrosation of MOR at 37°C in vitro.

In contrast the wild-type strain exhibited a low, but significant, nitrosating activity (50 nmol NMOR formed/mg protein/h).

This observation strongly suggests that cd1-NIR is involved in the catalysis of nitrosation by denitrifying bacteria.

Nitrosation catalysis by purified cd1-NIR

Purified cd1-NIR from Pseudomonas aeruginosa catalyzed in vitro nitrosation of MOR in the presence of DETC and nitrite. The specific activity determined by our standard nitrosation assay was 16.6 μmol NMOR formed/mg protein/h (equivalent to 1 μmol NMOR/1 nmol enzyme/h). Surprisingly, we observed no nitrosating activity when cd1-NIR was incubated with 2 mM NADH and 0.1% methylviologen, which are necessary cofactors in the measurement of nitrite-reductase activity of this enzyme. It is possible that NADH, a strong reducing agent, inhibited nitrosation. In addition, sequence analysis on 13 N-terminal amino acids of our nitrosating enzyme purified from Pseudomonas aeruginosa revealed complete homology with the cd1-NIR from Pseudomonas aeruginosa and peptide analysis also showed homology between the nitrosating enzyme and the cd1-NIR (Figure 1).

Inhibition of the nitrosating activity by antibodies raised against cd1-NIR

The polyclonal antibody raised against cd1-NIR immunoreacted with the nitrosating enzyme purified from Pseudomonas aeruginosa with an even higher sensitivity than the polyclonal antibody (Ab L901G) that we had raised against the purified nitrosating enzyme (10). The inhibition of both cd1-NIR and our nitrosating enzyme by Ab L901G and by Ab anti cd1-NIR was compared. The nitrosating activity of cd1-NIR was inhibited by both
antibodies. Using our nitrosation assay, 50% inhibition of both enzyme activities was observed at Ab/enzyme (cd\textsubscript{1}-NIR or nitrosating enzyme) ratios of 0.3 and 10 for Ab anti cd\textsubscript{1}-NIR and L901G respectively.

Spectral properties of the purified nitrosating enzyme

The UV and visible absorption spectra of the nitrosating enzyme purified from Paeruginosa (Figure 2) exhibited absorption maxima at 410 (Soret band), 525 and 635 nm with shoulders at 360 and 560 nm. This spectrum is similar to that reported for the oxidized form of cd\textsubscript{1}-NIR (13).

EPR detection of NO derived from the nitrosating enzyme in vitro

When 100 μM nitrosating enzyme was incubated with nitrite and DETC, an EPR spectrum was observed (Figure 3A) which showed a triplet typical of a [Fe(DETC)]\textsubscript{2}-NO ternary complex, with a g value of 2.035 (14,15). The [Fe(DETC)]\textsubscript{2} complex with NO is quite stable towards oxidation and allows the recording of NO accumulation in aqueous media. In addition, two weak EPR signals at g = 1.98 and 2.14 of unknown origin were also observed. When MOR was added to the assay, the typical signal of the [Fe(DETC)]\textsubscript{2}-NO complex disappeared (Figure 3B), suggesting that NO is used up for the nitrosation of MOR.

EPR detection of NO complexes in non-denitrifying nitrosating bacteria

Non-denitrifying bacteria such as Proteus morganii or Escherichia coli were shown to exhibit nitrosation activity (16). When the nitrosating enzyme (isolated from denitrifying bacteria) was replaced by a suspension of these bacteria, typical EPR signals of NO bound to an [Fe-S] protein complex at g = 2.041 and 2.015 (Figure 4A, C) were detected (17-19), without requiring the addition of DETC. These signals were reduced (Figure 4D) or disappeared (Figure 4B) when morpholine was added to the assay mixture. P.morganii displayed a 10-fold higher nitrosating activity than E.coli and thus produced greater amounts of NO or NO\textsuperscript{+}-like species at faster rates. Therefore, higher concentrations of MOR were required to dissociate the [Fe-S]-NO complex via formation of NMOR.

Discussion

Taken together, our data provide strong evidence that cd\textsubscript{1}-NIR is one of the enzymes responsible for the catalysis of nitrosation observed in bacteria such as Paeruginosa and N.mucosae. A mutant strain of P.stutzeri, with a t\textsubscript{n} insertion in the cd\textsubscript{1}-NIR structural gene, lacked its capacity to catalyze nitrosation. Peptide and N-terminal sequence analysis of the nitrosating enzyme purified from Paeruginosa revealed complete homology with the cd\textsubscript{1}-NIR from Paeruginosa (Figure 1). Spectral properties of the nitrosating enzyme are similar to those of the cd\textsubscript{1}-NIR (Figure 2). In addition, purified Paeruginosa cd\textsubscript{1}-NIR catalyzed \textit{in vitro} nitrosation of MOR in the presence of DETC. This nitrosating activity was inhibited by antibodies raised against the cd\textsubscript{1}-NIR or the purified nitrosating enzyme (10). Finally typical EPR signals of a [Fe(DETC)]\textsubscript{2}-NO ternary complex were observed when nitrosating enzyme was incubated with nitrite and DETC (Figure 3). Although further studies are needed to elucidate the underlying mechanism, we postulate that NO or NO\textsuperscript{+}-like species are formed by cd\textsubscript{1}-NIR as a nitrosation intermediate in denitrifying bacteria. More interestingly, large quantities of NO or NO\textsuperscript{+}-like species are also produced by enterobacteria, as observed by EPR studies (Figure 4). This confirms previous observations (20). Under aerobic conditions, NO is oxidized to form NO\textsubscript{2}; NO\textsubscript{2} exists in equilibrium with the potent nitrosating agents N\textsubscript{2}O\textsubscript{3} and N\textsubscript{2}O\textsubscript{4}, which react at neutral pH with secondary amines to form N-nitrosamines. A similar three-step mechanism has been shown to be responsible for macrophage-mediated nitrosamine formation, with the difference that the NO is generated from L-arginine via NO-synthase (21-23). It is thus possible that NO or NO\textsuperscript{+}-like species produced \textit{in vivo} by bacterial and mammalian enzymes during chronic bacterial infections and inflammatory processes, both known as cancer risk factors, play a role in human carcinogenesis, as recently discussed (24,25).

References


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