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Purification and characterization of the single-component nitric oxide reductase from *Ralstonia eutropha* H16

Rainer Cramm*, Anne Pohlmann, Bärbel Friedrich

Institut für Biologie/Mikrobiologie, Humboldt-Universität zu Berlin, Chausseestrasse 117, D-10115 Berlin, Germany

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Abstract Nitric oxide (NO) reductase was purified from *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) using a two step chromatographic procedure. Unlike the common NO reductases, the enzyme consists of a single subunit of 75 kDa which contains both high-spin and low-spin heme *b*, but lacks heme *c*. One additional iron atom, probably a ferric non-heme iron, was identified per enzyme molecule. Whereas reduced cytochrome *c* was ineffective as electron donor, NO was reduced at a specific activity of 2.3 μmol/min per mg of protein in the presence of 2-methyl-1,4-naphthoquinol.

Key words: Nitric oxide reductase; Heme-copper oxidase; Quinol oxidase; Denitrification; *Ralstonia eutropha*

1. Introduction

Bacterial nitric oxide (NO) reductases play a crucial role in the denitrification pathway [1–4]. The enzyme catalyzes the reduction of the toxic intermediate NO, which is formed by the dissimilatory nitrite reductase. The product of NO reduction, nitrous oxide, is finally reduced to dinitrogen by nitrous oxide reductase. Although there was doubt about NO as an obligate intermediate [2,5], recent investigations demonstrated that the absence of NO reductase was lethal for denitrifying cells [6–9], presumably due to the toxicity of NO. The prototype of NO reductase is represented by the NorCB heterodimer, which has been purified from various denitrifiers [10–15]. The NorB subunit was characterized as a membrane-bound protein which carries two *b*-type hemes and one non-heme iron per molecule. Most likely, NO is reduced at a dinuclear center consisting of a heme *b* in the high-spin state and the non-heme iron [16–18]. The DNA sequence of the NO reductase gene of *Pseudomonas stutzeri* provided the molecular basis for the perception that the putative tertiary structure of NorB resembles that of heme-copper oxidases [3,9]. Moreover, since conserved residues indicate a similar reaction mechanism, NO reductases are considered to be the evolutionary precursors of heme-copper oxidases [19–21]. According to this view, the heme *c* containing NorC subunit of NO reductases would be an analogue of subunit II of heme-copper oxidases.

*Corresponding author. Fax: (49) (30) 20938102.
E-mail: rainer.cramm@rz.hu-berlin.de

Abbreviations: NTA, nitrilotriacetic acid; PMS, phenazine methosulfate; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; EPR, electron paramagnetic resonance

In previous investigations, we have shown by sequence analysis and investigation of mutants that denitrifying cells of *Ralstonia eutropha* H16 (formerly *Alcaligenes eutrophus*, [22]) contain two NO reductase isoenzymes, designated NorB and NorZ, which both differ from the NorCB prototype [7]. NorB and NorZ are clearly homologues of the large subunits of the known NO reductases, but both proteins carry a N-terminal extension which has the potential of forming a large periplasmic loop. A gene with coding capacity for a NorC-like protein has neither been detected in the vicinity of *norB* nor *norZ*. The apparent absence of a second subunit raised the question whether the NO reductases from *R. eutropha* represent a single-component enzyme. If this is the case, the transfer of reduction equivalents should proceed differently in this type of NO reductase, pointing to a specific role of the N-terminal extension in the electron transfer process. In this work, we have purified the NorB from *R. eutropha* and give evidence that the protein represents a novel type of quinol oxidase.

2. Materials and methods

2.1. Strains and plasmids

Plasmid pGE423 was used for overexpression of NorB in the strain *R. eutropha* HF420 [7], which is a NorB/NorZ negative derivative of the wild-type H16. For construction of pGE423, a 3.2 kb *Bsr*BI fragment of pCH510 [7], containing the *norB* gene, was cloned into the *Eco*RV-linearized vector pBluescript SK+ (Stratagene), yielding plasmid pCH538. A 400 bp fragment was amplified from pCH538 with the two primers PNDE: 5′-GGAATTCCATATGGGCTCATATCGCAGACT-3′ and PBAM: 5′-TTCGAGACGGTCAGCACGCT-3′. PNDE generates a *Nde*I site at the start codon of *norB*. The PCR fragment was digested with *Nde*I-*Bam*HI and cloned into the *Nde*I-*Bam*HI-linearized vector pET22b+ (Novagen) to give pCH686. The complete *norB* gene was restored by cloning a 2.3 kb *Bam*HI-*Hin*dIII fragment from pCH538 into the *Bam*HI-*Hin*dIII-linearized plasmid pCH686, yielding pCH687. A 100 bp fragment was amplified from pCH538 with the two primers PECO: 5′-GGCTGGTGCTGATCGGG-3′ and PBGL: 5′-GCGCAGATCTCTGCGCGGCGTTCAGGCGTCC-3′. PBGL generates a *Bgl*II site instead of the TAA stop codon of *norB*. The PCR fragment was digested with *Eco*RI-*Bgl*II and cloned into the *Eco*RI-*Bgl*II-linearized expression vector pQE60 (Qiagen), yielding pCH624. In this construct, the 3′-*norB* coding sequence is extended by six histidine codons (*norB*-6H). A 190 bp *Eco*RI-*Hin*dIII fragment from pCH624 was cloned into the *Eco*RI-*Hin*dIII-linearized plasmid pCH687, which replaced the native 3′-coding sequence of *norB* by the modified *norB*-6H sequence. From this plasmid (pCH688), a 2.3 kb *Xho*I-(Klenow-polished) *Nde*I fragment containing the entire *norB*-6H gene was isolated and cloned into the *Nde*I-*Sna*BI-linearized plasmid pCH591, yielding pCH691. pCH591 carries the promoter of the soluble hydrogenase of *R. eutropha*. The detailed construction of pCH591 will be described elsewhere (L. Kleihues, O. Lenz, M. Bernhard and B. Friedrich, in preparation). A 2.6 kb *Hin*dIII fragment, encompassing the SH promoter region and the *norB*-6H gene, was cloned into the *Hin*dIII-linearized broad host range vector pVK101 [23], yielding plasmid pGE423.

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2.2. Purification of NorB

Strain HF420 bearing plasmid pGE423 was grown at 30°C in a 70 l fermenter (Biostad D, Braun, Germany) filled with 50 l mineral salts medium containing 0.2% (w/v) fructose and 0.2% (w/v) glycerol (FGN medium). When the culture reached an optical density of 6–7 at 436 nm, aeration was stopped and sodium nitrate was added to a final concentration of 10 mM. Cells were harvested by continuous centrifugation at 10 000×*g* and washed in 50 mM sodium phosphate buffer (pH 7.5). The cell pellet was immediately frozen in liquid nitrogen and stored at −20°C. About 5 g of cells (wet weight) was obtained per liter of medium. All purification steps were carried out on ice or at 4°C. Cells (20 g) were suspended in 10 ml of 50 mM sodium phosphate buffer (pH 7.5) containing 5 ml protease inhibitor cocktail (Sigma). Solid DNAse I was added and cells were broken by passing them three times through a French press cell at 1100 psi. The cell debris was removed by two centrifugation steps for 1 h at 6000×*g*. The supernatant was centrifuged for 1 h at 48 000×*g*. The pelleted membranes were resuspended in 50 mM sodium phosphate buffer (pH 8.0) containing 50 mM NaCl, 1 mM 4-(2-aminoethyl)-benzolsulfonylfluoride to give a final concentration of 10 mg protein/ml. *n*-Dodecyl β-D-maltoside was added to a final concentration of 1% (w/v) from a 10% (w/v) stock solution. After 1 h shaking on ice, solubilized components were separated by centrifugation for 1 h at 48 000×*g*.

The clear supernatant was applied to a 10 ml C/10 column (Pharmacia) packed with 2 ml of a Ni-nitrilotriacetic acid (NTA) silica matrix (Qiagen), which was previously equilibrated with two column volumes of 50 mM sodium phosphate buffer (pH 8.0) containing 300 mM NaCl, 20 mM imidazole, 0.1% (w/v) *n*-dodecyl β-D-maltoside. After washing with the same buffer, a step gradient with 50 mM imidazole was applied. In a final step, residual proteins were eluted in a linear gradient from 50 to 200 mM imidazole. Fractions containing NorB were immediately diluted 10-fold with 50 mM sodium phosphate buffer (pH 7.0) containing 0.1% (w/v) *n*-dodecyl β-D-maltoside and concentrated in Amicon 30K centriprep concentrators to about 1 ml. This step removed most of the imidazole. Contaminating impurities were removed by cation exchange chromatography on a PO-ROS-HS-M column (PerSeptive Biosystems) equilibrated with 50 mM sodium phosphate buffer (pH 7.0), containing 50 mM NaCl, 0.1% (w/v) *n*-dodecyl β-D-maltoside, 0.01% (w/v) phosphatidylcholine. NO reductase was eluted in a buffered linear salt gradient from 50 to 500 mM NaCl. Pooled fractions were concentrated in Amicon 30K centriprep concentrators to contain 1–2 mg/ml protein. The concentrated enzyme solution was stabilized by the addition of 0.1 mM ethylenediaminetetraacetic acid, 10% (w/v) glycerol and stored at −70°C.

2.3. Spectroscopy

Optical absorption spectra were measured with a Hitachi U-2000 spectrophotometer. Electron paramagnetic resonance (EPR) spectra were obtained with a Bruker ESP-300E spectrometer, equipped with an Oxford helium flow cryostat. Atomic absorption spectroscopy was performed on a Perkin-Elmer PE5100/Zeeman spectrometer.

2.4. Enzyme assays

NO reductase activity was assayed at 30°C as described previously [7] using a Clark electrode. The reaction mixture (2 ml) contained 50 mM sodium phosphate buffer (pH 7.0), 20 mM D-glucose, 10 U glucose oxidase and 250 U catalase. NO reduction was measured using 10 μmol ascorbate plus 0.25 μmol phenazine methosulfate (PMS) or *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD). NADH-dependent NO reduction was measured using 0.25 μmol NADH and 0.35 μmol of one of the following compounds: 2-methyl-1,4-naphthoquinone, tetramethyl-1,4-benzoquinone, 2,3-dimethoxy-5-methyl-1,4-benzoquinone and horse heart cytochrome *c*. Quinols were formed from the corresponding quinones in the presence of 40 U diaphorase from *Chlostridium kluyveri*. Cytochrome *c* was reduced in the same way as the quinones. After an incubation period of 5 min, NO-saturated buffer (100 nmol NO) was added. The reaction was started by the addition of NO reductase (15–30 μg).

2.5. Analytical procedures

Protein concentrations were determined according to Lowry [24]. Cell fractions and purified protein were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [25] using a 10% polyacrylamide gel. A benchmark protein standard (Gibco BRL) was used to determine the molecular masses. Gels were stained for protein with Coomassie brilliant blue G250. Nitrous oxide was measured by gas chromatography as described previously [7].

3. Results

3.1. Overexpression of NO reductase in R. eutropha

To facilitate purification, the C-terminus of NorB was genetically engineered to contain six additional histidine residues. The modified gene (*norB*-6H) was cloned under control of the promoter of the soluble hydrogenase of *R. eutropha* and the recombinant plasmid was transferred into the *R. eutropha* mutant HF420 which lacks the NorB and NorZ proteins. For a controlled expression of *norB*-6H, the cells were cultivated on fructose and glycerol, yielding a diauxic growth. The promoter of the hydrogenase is derepressed during energy limitation on poor carbon sources like glycerol [26]. However, preliminary experiments indicated that NorB was produced as an inactive protein under these conditions. Therefore, the culture was shifted to denitrifying conditions after fructose was exhausted. The expression of active NO reductase was monitored by determination of the reaction product, nitrous oxide.

3.2. Purification of NO reductase

A summary of the purification of NO reductase is given in Table 1. NO reductase was solubilized from the membrane fraction in the presence of the detergent, *n*-dodecyl β-D-maltoside. About 80% pure protein was obtained after immobilized-metal ion affinity chromatography on the Ni-NTA column. The NO reductase eluted as a single peak above 150 mM imidazole, as monitored by the absorption at 415 nm. Since the protein precipitated slowly in the presence of high concentrations of imidazole, the eluate was diluted 10-fold and subsequently concentrated to a final concentration of 2 mg/ml. Contaminating proteins were removed by cation exchange chromatography. A minor fraction of NO reductase and most of the contaminating proteins did not bind to the column. Bound NO reductase was eluted in a linear salt gradient above 200 mM NaCl. Since the protein tends to smear on the

Table 1
Purification of NorB

Purification step	Total protein (mg)	Specific activity[a] (nmol NO/mg/min)	Yield (%)
Membranes	550	507	100
Membranes[b]	290	510	53
Silica-NTA	3.1	1 950	2.2
POROS-HS	1.9	2 790	1.9

[a]Measured with ascorbate+PMS as electron donor.
[b]After solubilization in 1% *n*-dodecyl β-D-maltoside for 2 h.

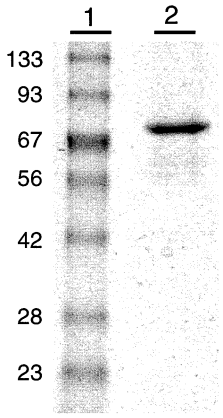

Fig. 1. SDS-PAGE of purified NO reductase. Lane 1: standard proteins, the molecular masses (kDa) are shown on the left side. Lane 2: 2 μg of purified NO reductase.

column, fractions were checked for purity by SDS-PAGE before pooling.

Purified NO reductase was homogenous on SDS-PAGE, showing a single band with an apparent molecular mass of 75 kDa (Fig. 1). The discrepancy of this size and the molecular mass of 84.5 kDa, as calculated from the amino acid sequence [7], may be due to the hydrophobicity of the protein. Similar observations are reported for the large subunit of the NO reductases from other denitrifiers [13–15,17].

3.3. Spectroscopic analysis

Fig. 2A shows the optical spectra of the oxidized (as isolated) and the reduced (dithionite) NO reductase. The maxima in the Soret region at 414 nm in the oxidized state and at 423 nm in the reduced state are typical for a hemoprotein. The oxidized enzyme displayed a broad peak with a maximum at 534 nm and a shoulder at around 565 nm. The sharpening and increase in intensity upon reduction, with peaks at 527 and 557 nm in the β- and α-band region, suggest the presence of low-spin heme *b*. An additional weak signal was observed at approximately 599 nm in the reduced enzyme. Nevertheless, a clear decrease in absorbance at 580 nm upon reduction is visible in the reduced minus oxidized difference spectrum (Fig. 2B). A similar decrease in absorbance has been observed with all NO reductases purified so far and was assigned to a charge transfer transition of high-spin heme *b* [13]. The lack of a signal at around 551 nm in the reduced enzyme suggests that heme *c* is absent from the NO reductase of *R. eutropha*.

The EPR spectrum of the NO reductase, as isolated, shows typical features of a protein containing high-spin and low-spin hemes (Fig. 3). A signal at $g = 6.0$ was weak at 10 K (A) but clearly visible when lowering the temperature to 4 K (B). This signal is most likely due to high-spin heme iron. A signal at $g = 4.3$ could be derived from adventitious iron. The $g = 2.01$ signal may be due to a non-metallic radical, however, such a signal may also be derived from a non-heme high-spin ferric iron. Two out of the four remaining signals can be ascribed to a low-spin heme, most likely with parameters $g_z = 2.96$, $g_y = 2.28$. Another broad and very weak signal at around $g = 1.4$ (data not shown) was observed at a high instrument sensitivity, which may represent the g_x component of a low-spin heme. The origin of the signals at $g = 2.55$ and at $g = 2.07$ is uncertain. A signal at $g = 2.5$ has been assigned to the g_z component of a low-spin species in the cytochrome *bd* ubiquinol oxidase from *Escherichia coli* [27].

Atomic absorption spectroscopy showed the presence of 2.8 Fe atoms per protein molecule. Copper was detected at a concentration of 0.2 atoms per protein molecule. Based on the spectroscopic properties, one can assume that the enzyme contains one high-spin heme and at least one low-spin heme per molecule. The low-spin species represents most likely a *b*-type heme, since neither a heme *c* signal was obtained in the optical spectra nor the amino acid sequence of NorB shows an appropriate sequence for binding of heme *c*. Furthermore, heme staining of the purified protein in SDS-PAGE (not shown) gave no evidence for covalently bound heme.

3.4. Kinetic properties

Table 2 shows the activity of purified NorB in the presence of different electron donors. Ascorbate-reduced PMS turned out to be the most effective electron donor for this enzyme. However, the specific activity was 2–10-fold lower than the PMS-dependent activities reported for the NO reductases of *P. stutzeri* and *Paracoccus denitrificans* [13,14]. When TMPD was used instead of PMS, the activity dropped by one order of magnitude. TMPD was used successfully with the NO reductase from *P. denitrificans* [17], but was reported to be ineffective with the enzyme from *P. stutzeri* [14]. Interestingly, the NO reductase of *R. eutropha* showed also quinol oxidase activity. In particular, reduced menadione led to an activity which is comparable to that obtained with reduced PMS. The activities with the quinols appeared to be mainly a function of

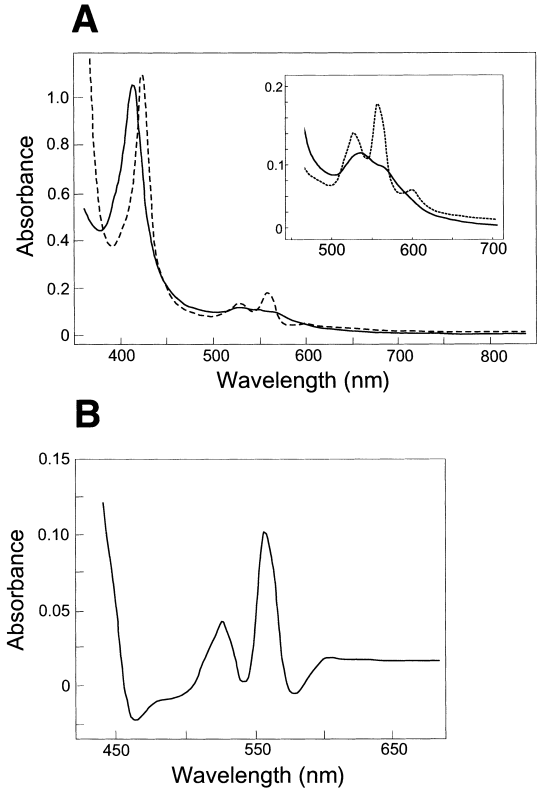

Fig. 2. Optical absorption spectra of purified NO reductase. A: Absolute spectra of the oxidized and dithionite-reduced (dashed line) states. The inset shows a magnification of the α- and β-band region. B: Dithionite-reduced minus oxidized difference spectrum.

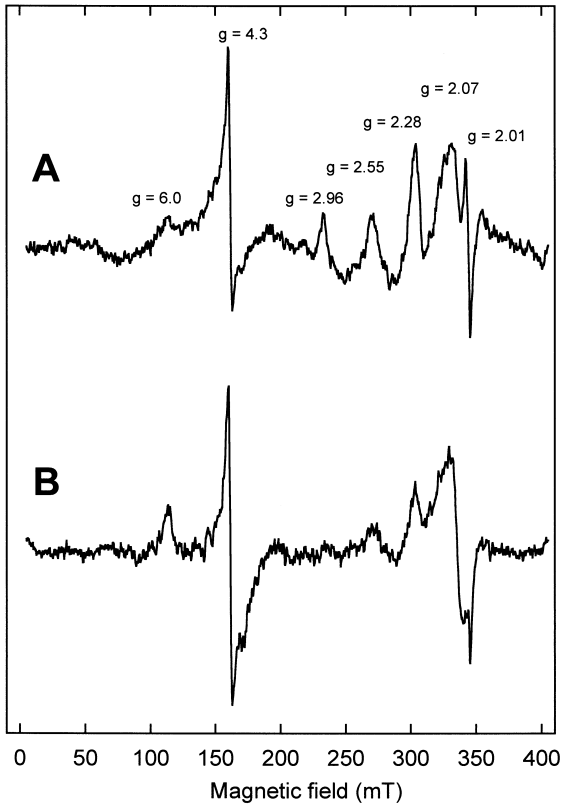

Fig. 3. X-band EPR spectra of purified NO reductase as isolated. Experimental conditions: frequency 9.650 GHz, modulation frequency 100 kHz, modulation amplitude 1.6 mT, time constant 327 ms, microwave power 40 mW (A) or 5 mW (B), temperature 11 K (A) or 4.1 K (B).

their redox potentials with $E_0' = -1$ mV (menadiol), +35 mV (duroquinol) and +162 mV (Q_0H_2) [28]. Quinol oxidation was not significantly decreased in the presence of up to 1 mM 2-heptyl-4-hydroxyquinoline-*N*-oxide (HOQNO). HOQNO, an inhibitor of electron transfer, acts at quinone-binding sites [29,30]. No activity was observed when either diaphorase or NADH was omitted from the reaction mixture. Comparatively high activities were obtained, however, in the presence of NADH and diaphorase when no quinone was added. Apparently, the NO reductase acts as an electron acceptor of diaphorase under these conditions. The reaction was blocked in the presence of horse heart cytochrome *c*, which is reduced by diaphorase but which is not oxidized by the NO reductase. Thus, unlike the common NorCB-type NO reductases, the physiological electron donor for the enzyme from *R. eutropha* is most probably a quinol, but not a cytochrome *c*.

4. Discussion

Analysis of the gene region encoding NorB of *R. eutropha* showed that the protein, although extended by a N-terminal domain, is homologous to the catalytic subunits of common NO reductases [7]. In particular, all residues which are considered as putative ligands of the prosthetic groups [20,21] are conserved. Therefore, the enzyme from *R. eutropha* should reveal spectroscopic properties similar to those of the large subunit of common NO reductases. Indeed, optical spectroscopy confirmed the presence of heme *b* and the absence of heme *c*, which is a constituent of the small subunit of common NO reductases. However, the EPR data available for the latter group of enzymes have been interpreted controversially. Whereas the low-spin species with parameters around $g_{xyz} = 1.3$, 2.3, 2.9 has been assigned to heme *c* in the NO reductases from *P. denitrificans* and *Paracoccus halodenitrificans* [13,15], an equivalent signal was assigned to heme *b* in the NO reductases from *P. stutzeri* [16]. Since a similar signal is also derived from the NO reductase of *R. eutropha*, which does not contain heme *c*, our data support the latter assignment. All NO reductases purified so far contain additional iron not derived from heme. The signals around $g = 2.00$ and/or $g = 4.3$ have been assigned to this non-heme iron [13,15]. However, in preparations from *P. stutzeri* [16] and *P. denitrificans* [17], this signal was absent. Thus, the $g = 2.01$ signal observed with the NO reductase from *R. eutropha* may result from an additional component. Although a signal in the $g = 2$ region may reflect a contamination by a [3Fe4S] cluster [16,31], atom absorption spectroscopy of NorB indicated no elevated iron content.

The most intriguing feature of the NO reductase of *R. eutropha* is the presence of a N-terminal extension of approximately 280 amino acids. Obviously, the ability for quinol oxidation is a property of this extra domain. Indeed, a mutant containing a NO reductase which is devoid of this domain is unable to denitrify (R. Cramm and B. Friedrich, unpublished data). Since NO reductases are considered as distant relatives of both quinol oxidases and cytochrome *c* oxidases [16,20,21,32], the existence of a quinol-oxidizing NO reductase seems to be not surprising. Nevertheless, no homology is apparent between the extension of NorB and the quinol-oxidizing subunit of terminal oxidases. Thus, the NO reductase from *R. eutropha* may represent a novel type of quinol-oxidizing enzyme.

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Table 2
NO reduction of NorB with various electron donors

Electron donor	Specific activity (nmol NO/mg/min)
NADH	1 530
NADH+2-methyl-1,4-naphthoquinone (menadione)	2 270
NADH+tetramethyl-1,4-benzoquinone (duroquinone)	780
NADH+2,3-dimethoxy-5-methyl-1,4-benzoquinone (Q_0)	160
NADH+cytochrome *c* (horse heart)	< 1
TMPD+ascorbate	220
PMS+ascorbate	2790

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