Table I. ^{31}P NMR Characteriziation of Deoxynucleoside Phosphorothioamidites

compd	base (B)	amidite	R^3	^{31}P NMR (δ)[a]
3a	T	pyrrolidinyl	2,4-dichlorobenzyl	164.8, 161.8
3b	T	pyrrolidinyl	4-chlorobenzyl	164.2, 161.0
3c	T	dimethylamino	4-chlorobenzyl	172.3, 170.5
3d	T	dimethylamino	2,4-dichlorobenzyl	172.1, 170.4
3e	C^{Bz}	pyrrolidinyl	2,4-dichlorobenzyl	165.1, 162.6
3f	C^{Bz}	pyrrolidinyl	4-chlorobenzyl	161.8, 159.9
3g	C^{Bz}	dimethylamino	4-chlorobenzyl	171.9, 170.7
3h	C^{Bz}	dimethylamino	2,4-dichlorobenzyl	172.0, 171.0
3i	A^{Bz}	pyrrolidinyl	2,4-dichlorobenzyl	163.8, 162.7
3j	A^{Bz}	pyrrolidinyl	4-chlorobenzyl	163.5, 162.3
3k	A^{Bz}	dimethylamino	4-chlorobenzyl	171.8, 170.9
3l	A^{Bz}	dimethylamino	2,4-dichlorobenzyl	171.7, 170.9
3m	G^{iB}	pyrrolidinyl	2,4-dichlorobenzyl	163.9, 160.9
3n	G^{iB}	pyrrolidinyl	4-chlorobenzyl	163.4, 161.6
3o	G^{iB}	dimethylamino	4-chlorobenzyl	171.5, 169.5
3p	G^{iB}	dimethylamino	2,4-dichlorobenzyl	171.9, 169.6

[a] ^{31}P NMR were recorded in $CDCl_3$ on a Brucker WM-250 with 85% aqueous H_3PO_4 as external standard.

phorodithioate linkages proceeds according to the reaction sequence outlined in Figure 1, part B. Synthesis begins by reacting a dry acetonitrile solution of compounds **3a-p** (10 equiv) and tetrazole (50 equiv) with 1 μmol of **4** for 30 s (step i) followed by a 400-s oxidation with 5% sulfur in pyridine:carbon disulfide (1:1, v/v, step ii).[14] Coupling is performed twice to ensure high yields (greater than 98%). Acylation of unreactive compound **4** (step iii), detritylation (step iv), and various washes are the same as those described previously for synthesizing natural DNA from deoxynucleoside phosphoramidites.[15,16] Multiple repetitions of this cycle then lead to the synthesis of DNA containing exclusively phosphorodithioate linkages or, when used in combination with deoxynucleoside phosphoramidites, to oligodeoxynucleotides bearing both phosphorodithioate and phosphate internucleotide bonds.

Synthetic oligodeoxynucleotides are isolated free of protecting groups via a two-step protocol (thiophenol:triethylamine:dioxane, 1:1:2, v/v/v for 24 h followed by concentrated ammonium hydroxide for 15 h) and then purified to homogeneity by standard procedures (polyacrylamide gel electrophoresis and reverse phase HPLC).[17,18] ^{31}P NMR spectra (Figure 2B) of phosphorodithioate DNA indicates that oligonucleotides synthesized by using this approach contain exclusively phosphorodithioate internucleotide linkages. No hydrolysis of these dithioates to phosphorothioates (^{31}P NMR δ 56) or phosphate is observed.

Although two methods have been reported for synthesizing DNA having phosphorodithioate internucleotide linkages, these procedures suffer from substantial limitations. In one case, deoxynucleoside 3'-*O*-bis(diisopropyl)amidites are used to synthesize phosphorodithioate dinucleotides which are then incorporated into DNA.[8,9] The approach, however, lacks versatility as 16 synthons are required in order to introduce a dithioate linkage into any DNA sequence. Moreover deoxynucleoside diamidites having sufficient reactivity for efficient internucleotide bond formation are easily hydrolyzed and therefore difficult to incorporate into polynucleotide synthesis procedures. The other approach, which utilizes deoxynucleoside 3'-*N*,*N*-diisopropylphosphorothioamidites for preparing dinucleotides,[11] is also unsatisfactory for repetitive synthesis on supports. This is because these synthons are relatively inert and are only activated by strong acids such as pyridinium tetrafluoroborate—a reagent that leads to detritylation and side reactions due to long activation times.

The procedures outlined here are completely compatible with current methods for synthesizing natural DNA on silica supports.[16] Thus relatively simple mononucleotide synthons can be used for introducing phosphorodithioate and natural internucleotide linkages in any predetermined sequence. So far pentadecamer homopolymers containing up to 14 dithioate linkages, lac and cro operators with multiple dithioates at defined sites, and a cro operator segment containing 17 contiguous dithioates have been synthesized.[10] We anticipate that this approach using deoxynucleoside phosphorothioamidites as synthons will be the method of choice for preparing dithioate containing DNA.

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Evidence for a Copper-Nitrosyl Intermediate in Denitrification by the Copper-Containing Nitrite Reductase of *Achromobacter cycloclastes*

C. L. Hulse and B. A. Averill*

Department of Chemistry, University of Virginia Charlottesville, Virginia 22901

J. M. Tiedje

Department of Crop and Soil Sciences Michigan State University East Lansing, Michigan 48824

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Denitrification[1] is the microbial process by which nitrite is reduced to dinitrogen (eq 1). It is an important component of

$$NO_3^- \rightarrow NO_2^- \rightarrow N_2O \rightarrow N_2 \quad (1)$$

the global nitrogen cycle that is responsible for regulating the amount of fixed nitrogen available for plant growth.[2] The key step in the pathway, the reduction of nitrite to gaseous nitrogen oxides, is catalyzed by two classes of nitrite reductase, containing either a heme cd_1 unit or copper. The mechanism of nitrite reduction has been extensively investigated in systems known to utilize heme cd_1-containing nitrite reductases. These investigations have demonstrated (i) that nitrite and nitrous oxide are free intermediates in the denitrification pathway[1] and (ii) in the *Pseudomonas stutzeri* enzyme at least (and by implication in the other cd_1 enzymes) that N_2O formation occurs via nucleophilic attack of a second nitrite molecule[3-6] on an enzyme-bound nitrosyl intermediate[7,8] ($E{\cdot}NO^+$) derived from nitrite via a dehydration reaction. Inasmuch as hemes and heme enzymes readily form NO adducts under a variety of conditions,[9-12] while no well-

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Table I. Effects of Varied N_3^- and NH_2OH on the N_2O Produced from Denitrification (N_2O-46) and Nitrosation (N_2O-45) in Cell-Free Extracts of *Achromobacter cycloclastes*[a]

	denitrification (N_2O-46)		nitrosation (N_2O-45)	
control	50.8[b]	50.3	1.71	1.72
		$[N_3^-]$, mM		
1	26.2	45.0	2.30	1.95
10	25.0	18.5	1.61	1.13
100	9.24	7.06	3.51	3.09
		$[NH_2OH]$, mM		
1	1.93	1.92	23.9	24.3
10	0.67	0.56	178	140
100	0.78	0.66	198	194

[a] Experimental conditions: 50 mM succinate as reductant and 0.1 mM $[^{15}N]NO_2^-$ in 50 mM MES and 10 mM EDTA, pH 6.2, 3.0 mL total volume with 300 μL of crude extract (11 mg protein/mL); samples and sterile controls (autoclaved 15 min at 200 °C) incubated 1 h at 25 °C. Cell-free extract of *A. cycloclastes* prepared by sonication (3 × 5 min) and filtration through 0.22 μM millipore filter units. Gas samples, 500 μL, were analyzed with a HP5995C GC/MS system fitted with a Porapak Q column and operated in the SIM mode. [b] Millions of counts. Separate columns indicate results of duplicate experiments.

characterized Cu nitrosyls have been reported,[13-15] it remains to be demonstrated whether the Cu-containing nitrite reductases[16-21] exhibit chemistry parallel to that of the heme cd_1 systems. We report herein the results of isotope exchange and trapping experiments that demonstrate the existence of an enzyme-bound nitrosyl intermediate in the pathway of denitrification in *Achromobacter cycloclastes*, a species which utilizes a copper-containing nitrite reductase. This constitutes the first evidence that the mechanism of nitrite reduction by the copper-containing systems is similar to that of the heme cd_1 enzymes.

The existence of a nitrosyl intermediate was demonstrated by incubating cell-free extracts of *Achromobacter cycloclastes* with varying amounts of N_3^- and NH_2OH at a constant $^{15}NO_2^-$ concentration. The results are summarized in Table I. Trapping of an E·$^{15}NO^+$ intermediate with $^{15}NO_2^-$ (denitrification) should produce $^{15}N^{15}NO$ (N_2O-46), while reaction with a ^{14}N-containing nucleophile (nitrosation) should give ^{14}N-^{15}NO (N_2O-45). As shown in Table I, increasing $[N_3^-]$ results in a decrease in the relative amount of N_2O-46 formed, consistent with partitioning of the E–$^{15}NO^+$ intermediate to a alternative product (nitrosation). The total amount of N_2O produces steadily decreases with increasing $[N_3^-]$, suggesting that N_3^- is inhibiting formation of the E–$^{15}NO^+$ intermediate in addition to reacting with it.

Table II. Effects of Nucleophiles on NO Production by Purified Nitrite Reductase from *Achromobacter cycloclastes* as Measured by Gas Chromatography[a]

	NO produced[b]
	$[N_3^-]$, mM
0	1440 (20)
10	1240 (70)
100	857 (6)
	$[NH_2OH]$, mM
0	1540 (300)
10	1510 (380)
100	1750 (132)

[a] Experimental conditions: 5 mM NADH, 0.1 mM PMS and 1.0 mM NO_2^- in 50 mM MES and 10 mM EDTA, pH 6.2, 3.0 mL total volume with 0.3 μg purified nitrite reductase (ref 22); samples incubated 20 min at 25 °C. Gas samples, 125 μL, analyzed with a Perkin-Elmer 910 gas chromatograph fitted with a Porapak Q column and electron capture detector, operating under standard running conditions with a argon–methane (95%–5%) carrier gas. [b] Thousands of counts, n = 3; standard deviations in parentheses.

This conclusion is supported by experiments on the purified nitrite reductase from *A. cycloclastes*,[22] which produces only NO from NO_2^- at low $[NO_2^-]$ (vide infra). As shown in Table II, N_3^- is an efficient inhibitor of NO production from NO_2^-, indicating that it may also compete directly with NO_2^- for the Cu site. The net result of these two effects is an increase in the relative amount of the nitrosation product, N_2O-45, with a decrease in the overall amount of N_2O produced, as observed in Table I.

The effect of hydroxylamine as a trapping agent is also shown in Table I. With increasing $[NH_2OH]$, denitrification in cell-free extracts of *Achromobacter cycloclastes* is completely abolished, while nitrosation is stimulated such that N_2O production is ca. 4× that of the control. Unlike N_3^-, NH_2OH has no effect on NO production by purified nitrite reductase (Table II). The stimulatory effect on N_2O production suggests that NH_2OH is more effective than NO_2^- in trapping the E–NO^+ intermediate.

$H_2{}^{18}O$ exchange experiments have been crucial in establishing the mechanism of the heme cd_1 enzymes[3-6] and were also performed on cell-free extracts of *Achromobacter cycloclastes*. Under the conditions of the experiments (0.1 mM NO_2^-, 10 mM succinate, >19% $H_2{}^{18}O$ in 50 mM HEPES, pH 7.3), no detectable ^{18}O was incorporated into N_2O (under conditions where ≤1% isotope equilibration[3] would have been observable). The absence of exchange can be rationalized based on either thermodynamic grounds (if the equilibrium between nitrite and the nitrosyl intermediate lies far to the right) or on kinetic grounds (if the rate of reaction of the E·NO^+ species with nitrite is substantially greater than that of reaction with H_2O under the conditions of the experiment.)

The results of these experiments demonstrate that, as with the heme cd_1 systems, the copper system produces N_2O via a bound nitrosyl intermediate, E–NO^+; this is presumably a cuprous nitrosyl, Cu^+–NO^+, of which there are no structurally characterized synthetic examples.[13-15] Despite their mechanistic similarity, the chemical characteristics of the intermediates in the cd_1 and Cu systems are quite different. In both systems, a nitrosyl intermediate is capable of being trapped with nucleophiles such as N_3^- and NH_2OH. With the Cu system, NH_2OH is a far more efficient trapping agent than either N_3^- or H_2O and results in an enhancement of nitrosation, a result not seen in the heme cd_1-containing systems.[3-5] Further studies are in progress to examine the reasons for the absence of $H_2{}^{18}O$ exchange and the role of the second nitrite in N_2O formation in this system.

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