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Mechanism for Nitrosation of 2,3-Diaminonaphthalene by *Escherichia coli*: Enzymatic Production of NO Followed by O_2-Dependent Chemical Nitrosation

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The mechanism by which *Escherichia coli* can catalyze the nitrite-dependent nitrosation of 2,3-diaminonaphthalene (DAN), with formation of the corresponding fluorescent triazole, was studied. The reaction was dependent on production of a gaseous compound which can nitrosylate DAN upon contact with air. This compound was identified as nitric oxide (NO), and the kinetics of NO and triazole production are reported. NO and triazole were produced proportionally in a stoichiometric ratio, NO/triazole, of 1.4 to 1.7. Given the requirement for air, nitrosation of DAN probably proceeds via formation of the well-known strong nitrosylating agents N_2O_3 and N_2O_4 from NO. The parallel inhibition of NO and triazole production by azide and nitrate served to reinforce the link between nitrosation and nitrate reductase that had been established previously by others on genetic grounds.

Ralt et al. (17) recently demonstrated that the ability of *Escherichia coli* to catalyze nitrosation reactions could be measured sensitively by conversion of 2,3-diaminonaphthalene (DAN) to its corresponding fluorescent triazole, 1-[H]-naphtho[2,3-*d*]triazole. In addition, they showed that this reaction and the related nitrosation of morpholine were linked to nitrate reductase genes. Calmels et al. (5, 6) similarly linked the nitrosation of morpholine to nitrate reductase by genetic and biochemical means. Bacterial nitrosations are clearly of environmental and clinical interest, and so the mechanism of triazole formation by *E. coli* was studied. We show herein that triazole formation is dependent on the generation of nitric oxide from nitrite.

MATERIALS AND METHODS

Reagents. DAN was obtained from Sigma Chemical Co. and used without further purification because it was found to have a very low background fluorescence at 450 nm in the nitrosation assay because of contaminants. Its nitrosylated product, 1-[H]-naphtho[2,3-*d*]triazole (triazole), was generously provided by S. R. Tannenbaum. All other salts and chemicals were obtained commercially. The liquid broth (LB) medium was prepared as described by Miller (14).

Bacteria. *E. coli* K-12 strain RF1005 was provided by D. Ralt, and wild-type strain JM101 was provided by R. F. Schleif. Bacteria were grown anaerobically at 37°C without shaking in filled bottles on LB supplemented with 100 mM KNO_3, as described by Ralt et al. (17). In some cases the nitrate was omitted. For aerobic growth, the culture bottles were filled to 20% of their capacity and shaken in a New Brunswick rotary shaker. Cells were harvested by centrifugation at 4°C just as or just before cultures reached the stationary phase and washed twice at 4°C with phosphate-buffered saline (PBS), pH 7.3 (17). Finally they were suspended in PBS to the desired cell concentration.

Nitrosation of DAN. When triazole production was measured fluorometrically, the nitrosation reaction was carried out as described by Ralt et al. (17), except that strictly anaerobic conditions were maintained until the centrifugation step. Typically, 1.9 ml of cell suspension was placed in a 3.5-ml screw-top anaerobic vial sealed with a silicone rubber septum and sparged with Ar for 15 min. Gas-tight syringes were used to add 40 μmol of anaerobic sodium formate and 0.4 μmol of anaerobic DAN, and the reaction was initiated by injection of 50 μmol of anaerobic $NaNO_2$; the final volume was 2.0 ml. After an incubation period at 37°C, 1.5 ml was removed and clarified by centrifugation at room temperature for 5 min in an Eppendorf centrifuge (model 5412). The supernatant was diluted 10-fold with PBS to decrease the UV absorbance of nitrite to acceptable levels, and the triazole concentration of the resulting solution was determined by use of a Perkin-Elmer MPF-3 fluorescence spectrophotometer. The excitation wavelength was 375 nm and the observation wavelength was 450 nm in the monochromatic mode (17) and from 380 to 600 nm in the scanning mode. In some cases, 0.4 to 0.6 μmol of NO dissolved in buffer was substituted for nitrite.

Assay of NO and N_2O. NO was measured in experiments that paralleled the nitrosation assays by three methods: (i) by use of an anaerobic Clark-type oxygen electrode (Hansatech, model DW-1) at −0.7 V and 30 or 37°C (24); (ii) by gas chromatography (GC) of headspace gases with a Shimadzu model GC-9A unit with a thermal conductivity detector (2); and (iii) GC-mass spectrometry (GC/MS) of headspace gases with a Hewlett-Packard model 5992A machine (11). The root mean square noise level of the electrode corresponded to a change in NO concentration of about 2 μM, and the instability of the system over several minutes was equivalent to a change in NO concentration of about 0.2 μM. At −0.7 V, the ratio i_{NO}/i_{N_2O} was ≃100 and the response half-time was 3 to 4 s. For GC or GC/MS experiments, the anaerobic reaction mixture was constituted in a 9-ml serum bottle and shaken vigorously at 37°C. In some cases, 8 μmol of NO was substituted for the 50 μmol of nitrite. N_2O was measured by the GC or GC/MS method.

Protein. Cell protein was assayed by the Bio-Rad Laboratories (Bradford) method as described by Ralt et al. (17).

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TABLE 1. Effect of sparging with Ar on the yield of triazole[a]

E. coli strain	Nitrosation (nmol of triazole formed/mg of protein)		
	Without sparging	Sparged for 10 min after 1-h incubation	Sparged throughout 1-h incubation
RF1005	135	19	0
JM101	105	16	0

[a] Cells were grown on LB-nitrate, and the anaerobic reaction mixture contained 0.2 mM DAN, 20 mM formate, 25 mM $NaNO_2$, and cell protein at 1 mg/ml. Incubation at 37°C was terminated after 60 min.

RESULTS

Nitrosation and nitric oxide. Both of the *E. coli* strains studied performed the nitrosation of DAN much as described for strain RF1005 by Ralt et al. (17), except that formate was observed to have little influence on triazole yields under the anaerobic conditions of incubation. The reported ability of formate to boost triazole yields (17) was realized when reactions were run under the aerobic or microaerobic conditions used by Ralt et al. (17). Triazole was not detected in systems lacking nitrite in any case.

Triazole yields could be greatly diminished or abolished when the system was sparged with an inert gas during or at the end of the anaerobic incubation period (Table 1). This implies that nitrosation depends on generation of a gaseous product; it actually does not occur under anaerobic conditions but rather occurs largely or entirely only after the system has been exposed to air at the centrifugation step. Neither DAN nor triazole was removed from solution by the sparging. A gas that could perform in exactly the manner observed is NO, and so an attempt to detect its generation from nitrite by *E. coli* was undertaken in systems identical to those used for the nitrosation assay. As shown in Fig. 1, *E. coli* RF1005 generated NO for up to 30 min, with initial rates of about 10 nmol/min per mg of cell protein and maximum concentrations of 200 to 300 μM. Rates of N_2O production from nitrite were much smaller, about 0.07 nmol/min per mg of cell protein. Production of NO and N_2O was absolutely dependent on the presence of nitrite but unaffected by DAN, at least in the range from 0 to 0.2 mM. Carbon dioxide was also formed by the system, but it was not relevant to the nitrosation of DAN. N_2O production from NO over a period of 40 min was about 0.08 nmol/min per mg of cell protein. That is, the rates of N_2O production from nitrite and NO were the same or nearly so. The amount of NO used in these experiments (8 μmol) corresponded roughly to an equilibrium aqueous concentration of NO of 50 μM or about 1/4 of the maximum NO concentration shown in Fig. 1. Figure 1 also shows that there was a close correlation between NO production measured amperometrically and triazole production measured fluorometrically, with the NO/triazole ratios falling in the range 1.4 to 1.7. The importance of NO in the nitrosation of DAN was confirmed in experiments in which NO (400 nmol) dissolved in buffer was injected into systems lacking nitrite (with or without *E. coli*) toward the end of the anaerobic incubation period. In such experiments, triazole was formed with NO/triazole stoichiometries of 1.3 to 1.7, as might be expected from the data in Fig. 1. The chemistry of triazole formation seemed not to distinguish between added NO and NO formed in situ and seemed not to require cells. The presence of nitrate in the nitrosation system temporarily blocked both NO and triazole production (Fig. 1) until its conversion to nitrite. Nitrate at 12 mM blocked production for 10 to 12 min, whereas 25 mM nitrate blocked it for somewhat over 1 h. The disproportionate length of the inhibitory period at high nitrate concentrations is presumed to be related to decreased concentrations of formate, which is consumed in nitrate reduction.

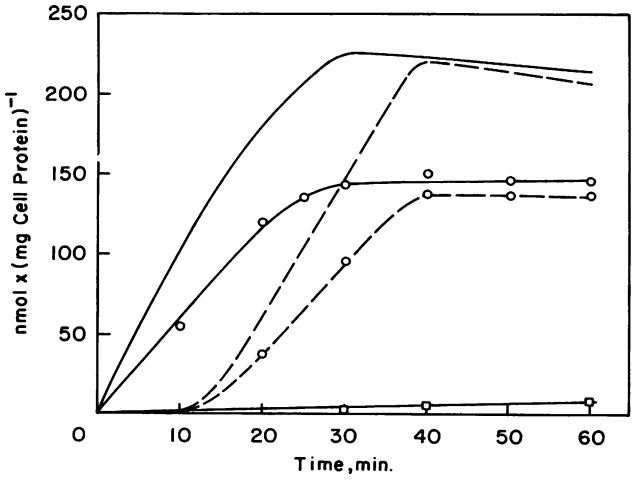

FIG. 1. Production of NO, triazole, and N_2O from nitrite by *E. coli* RF1005. Cells were grown on LB-nitrate, and the reaction mixture for the three assays was as described in Table 1, footnote *a*. Dissolved NO (—) was determined by the O_2 electrode from strip chart records; triazole (○) was determined by fluorescence; N_2O (□) was determined by GC. Solid lines, Without nitrate; dashed lines, with 12 mM KNO_3. Because the cell concentration was 1 mg of cell protein per ml, the ordinate also reads in micromolar. The slow decrease in NO level that occurred after 30 min was attributed largely to degassing.

TABLE 2. Inhibition of nitrosation and NO production by cyanide and azide[a]

Inhibitor	Concn (μM)	Decrease (%) in final concn	
		Triazole	NO
Cyanide	1,000	80	70
	5,000	100	100
Azide	0.3	56	50
	0.8	77	70
	1.0	84	90
	10	100	100

[a] *E. coli* RF1005 was grown on LB-nitrate. The reaction mixture for the nitrosation and O_2 electrode assays was as described in Table 1, footnote *a*, and incubations were terminated after 1 h. Data for NO were obtained with the O_2 electrode at 30°C. Rates and amounts of NO production at 30°C were similar to those at 37°C. The concentrations of triazole and NO in the absence of inhibitors were about 135 and 220 μM, respectively.

Nitrosation and NO production were strongly inhibited by azide and weakly by cyanide under anaerobic conditions (Table 2).

Physiological states. As shown in Table 3, *E. coli* cells grown in the absence of nitrate, and particularly aerobically, have diminished abilities to nitrosylate, in agreement with the general findings of Ralt et al. (17) and Calmels et al. (6). Note in this regard that decreases in NO production closely paralleled decreases in triazole production.

DISCUSSION

The results of this study show that catalysis of nitrosation of DAN by *E. coli* occurs largely or entirely by way of

TABLE 3. Effect of growth conditions on the ability of *E. coli* RF1005 to promote nitrosation and NO production[a]

Growth conditions	Nitrosation (nmol of triazole/mg of protein)	NO production (nmol/mg of protein)
Nitrate (100 mM), anaerobic	135	220
Nitrate (100 mM), aerobic	81	112
Anaerobic	24	40
Aerobic	0	0

[a] Cells were grown on LB as indicated. The reaction mixture for the nitrosation and O_2 electrode assays was as described in Table 1, footnote *a*, and incubations were terminated after 1 h. Data for NO were obtained with the O_2 electrode at 30°C.

well-known reactions of strong nitrosylating agents, such as N_2O_3 and N_2O_4, that arise from the rapid reaction of NO with O_2. The biologically significant process is NO production; nitrosation per se is chemical. The critical roles of NO and air in nitrosation were unexpected. It is interesting that *E. coli*, when adapted for nitrate respiration, can produce NO from nitrite at rates as much as 1/20 of those observed for NO production by typical denitrifying bacteria (12). The yields of triazole realized from nitrite (or added NO) were similar to those reported by Ralt et al. (17) and to yields of *N*-nitrosomorpholine reported by Calmels et al. (6) for *E. coli* A10. Because nitrosation reactions by *E. coli* are genetically and biochemically linked to nitrate reductase genes (5, 6, 17) and NO is linked to triazole production (this work), a plausible model for NO production by *E. coli* is the reduction of nitrite to NO as a secondary activity of nitrate reductase. The strong inhibition of NO production by azide during turnover and weak inhibition by cyanide are also consistent with involvement of a nitrate reductase of the respiratory or A type (1, 8–10, 15, 16, 18, 23). Inhibition by nitrate (6, 16; this study) is presumed to be due to its being preferred over nitrite as a substrate for nitrate reductase. We predict that some other bacteria that have been reported to catalyze nitrosation reactions (4–6) will also be found to generate NO from nitrite and, conversely, that organisms that can generate appreciable levels of NO may be expected to promote nitrosation reactions. It is interesting in this regard that *Paracoccus denitrificans*, when its denitrification apparatus had been fully induced, failed in preliminary studies to convert DAN to triazole and also maintained extremely low (<1 μM) steady-state levels of NO during anaerobic reduction of nitrite (X.-B. Ji and T. C. Hollocher, unpublished data). Extremely low steady-state levels of NO had been predicted for this organism (12).

The ability of *E. coli* to produce N_2O from nitrite has been linked by Smith (20) to nitrate reductase, and the rates of N_2O production observed with strain RF1005 were in agreement with those reported by Smith (20) for other strains. In fact, a large variety of microorganisms can slowly reduce nitrite to N_2O (3, 13, 19–21). The near identity of the rates of N_2O production from nitrite and NO by *E. coli* is consistent with the pathway nitrite → NO → N_2O and allows a chemical basis for linking nitrate reductase to nitrosation (5, 6, 17), N_2O production (20) and NO production (this study).

NO generated by *E. coli* from nitrite never exceeded 300 μM in spite of large excesses of nitrite and formate. The synthesis of NO would appear therefore to be self-limiting.

Because *E. coli* can be a moderately good source of but only a poor sink for NO, this kind of metabolism may have ecological implications. Nondenitrifiers might be responsible in part for the NO found in water columns and sediments (22; B. B. Ward and O. Zafiriou, Deep Sea Res., in press).

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