Vol. 187, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 31, 1992 Pages 135-139

AN APPARENTLY ALLOSTERIC EFFECT INVOLVING N_2O WITH THE NITROUS OXIDE REDUCTASE FROM *WOLINELLA SUCCINOGENES*

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Received June 22, 1992

SUMMARY. It was shown that k_{cat} for the benzyl viologen cation (BV^+)-N_2O oxidoreductase activity of nitrous oxide reductase from *Wolinella succinogenes* was 2-3 times greater at high N_2O concentrations than at low. This effect of N_2O on k_{cat} exhibited a titration curve implicating a single secondary binding site for N_2O with a K_d of 130-200 μM (K_m with respect to N_2O is about 2.5 μM). This work represents the first evidence of an apparently allosteric kinetic effect among nitrous oxide reductases. Its possible cause is discussed. BV^+ was generated in these kinetic studies by addition of sub-stoichiometric amounts of dithionite. This means of reduction proved to be superior to the photochemical generation of BV^+ that had been used previously with the enzyme. Mass spectrometric measurements suggested that the M_r of the subunit of the enzyme is about 95,500 rather than 88,000.

The reduced benzyl viologen (BV^+)-N_2O oxidoreductase activity of the heme- and Cu-containing nitrous oxide reductase from *Wolinella succinogenes* can exhibit both an initial turnover-dependent activation and subsequent turnover-dependent inactivation (1). The latter effect is common to most if not all nitrous oxide reductases (1-4). In recent experiments designed to determine accurately the K_m of nitrous oxide reductase for N_2O in the above reaction, we observed that k_{cat} was 2-3 times lower at 25-50 μM N_2O than it was at 2-25 mM N_2O. This difference could be attributed in large part to the absence of turnover-dependent activation at low N_2O concentrations. The ability of N_2O to active the enzyme at sufficiently high concentrations is described herein and possible causes are discussed. These studies were facilitated by a change in the assay system that afforded greater kinetic reproducibility and enzyme activity.

EXPERIMENTAL PROCEDURES. *Wolinella succinogenes* ATCC 29543 was grown under N_2O at 1 atm in a modification of Yoshinari's broth (1,5), and nitrous oxide reductase was purified by the method of Teraguchi and Hollocher (1) to a peptide purity of about 95%. Nitrous oxide reductase activity was measured spectrophotometrically at 600 nm and 25 °C by two variations on the BV^+-N_2O oxidoreductase assay of Kristjansson and Hollocher (6). The one involved the anaerobic photochemical generation of BV^+ to concentrations of 70-80 μM in presence of proflavin and triethanolamine (7). For the other, 90-100 μM BV^+ (A_{600} ~ 1) was generated from 200 μM oxidized BV by the injection of 10 μl of approximately 12.5 mM dithionite solution for a final dithionite concentration in the assay cuvette of about 50 μM. The volume of enzyme solution injected was typically 2.5-5.0 μl, and the assay mixtures were 2.5 ml in final volume. Cuvettes

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0006-291X/92 $4.00

were sealed with silicone rubber stoppers molded at Brandeis University or Teflon/silicone rubber septa (Pierce), and anaerobic injections were made by syringe through these seals. K_m with respect to BV^+ and N_2O (K_{mBV} and K_{mN2O}) were estimated from reaction progress curves (A_{600} vs. time) under conditions, respectively, where BV^+ or N_2O was reaction limiting (1). Protein was assayed by the bicinchoninic acid method (8) using reagents prepared by Pierce.

The subunit molecular weight of nitrous oxide reductase was estimated by laser desorption, time-of-flight mass spectroscopy (Lasermat, Finnigan-MAT) referenced to bovine serum albumin. The UV-absorbing matrix was sinapinic acid dissolved in water/acetonitrite/trifluoroacetic acid (30:70:0.1 v/v).

RESULTS. Table 1 illustrates the advantage of chemical (dithionite) reduction over photochemical reduction for studies of the kinetics of nitrous oxide reductase from *W. succinogenes*. Although K_{mBV} and K_{mN2O} values changed little, if at all, k_{cat} was routinely at least 50% greater when chemical reduction was used. K_{mBV} and K_{mN2O} were determined to be 1.5 ± 0.5 and 2.5 ± 0.5 μM, respectively, using chemical reduction and about 2 and 2.5 μM, respectively, using photochemical reduction. Both sets of K_m values are about two times smaller than the set originally reported by Teraguchi and Hollocher (1). Those earlier results were somewhat influenced, in retrospect, by turnover-dependent inactivation which was largely avoided in the present studies by use of somewhat greater enzyme concentrations. In using photochemical reduction to generate BV^+, it was found that k_{cat} decreased with increasing time of irradiation of the BV-containing cuvette. This result suggests that an inhibitor was generated photochemically. In the experiments portrayed in Table 1, cuvettes were irradiated only as long as was required to generate 80 μM BV^+ and no longer.

A difference of 2-3 fold in k_{cat} was observed between high and low N_2O concentrations (left and right half of Table 1). At high concentration, the enzyme showed turnover-dependent activation over a period of about 1.5 min, so that the final k_{cat} was 2-3 times greater than initial k_{cat} (1). Activation was not observed at the lowest N_2O concentrations used (25 μM). Thus, the process of activation accounted for much if not all, of the difference noted in Table 1. It would appear that N_2O can control the rate of its own reduction through events that activate the enzyme. Fig. 1 summarizes kinetic data over a range of N_2O concentrations. The one curve, k_{cat} vs. log[N_2O], resembles a sigmoidal titration curve; the other curve, log(f / (1 - f)) vs. log[N_2O],

TABLE 1

Comparison of kinetic parameters for assay of nitrous oxide reductase from *W. succinogenes* using photochemical means dithionite to generate BV^+. Temperature was 20°C and the enzyme concentration was about 200 ng x ml^{-1} or 2.1 nM subunits. All data were obtained on the same day with the same enzyme preparation.

Reducing system	$[N_2O]_i$ = 25 mM; $[BV^+]_i$ = 80-100 μM				$[N_2O]_i$ = 25 μM; $[BV^+]_i$ = 80-100 μM		
	K_{mBV}	k_{catN2O}[a]	k_{catBV}[a]	k_{catBV}/K_{mBV}	K_{mN2O}	k_{catN2O}[a]	k_{catN2O}/K_{mN2O}
	μM	s^{-1}	s^{-1}	$M^{-1}s^{-1}$	μM	s^{-1}	$M^{-1}s^{-1}$
Photochemical	2.0	85	170	8.5×10^7	2.4	33	1.4×10^7
Dithionite (50 μM)	1.3	135	269	2.1×10^8	2.5	59	2.4×10^7

[a] $k_{catBV} = 2\ k_{catN2O}$ by virtue of the stoichiometry of the BV^+-N_2O oxidereductase reaction. Alternative units for k_{cat} are mo substrate x s^{-1} x(mol of enzyme subunit)$^{-1}$. For systems containing 25 mM N_2O, k_{cat} refers to final reaction rates after turnov dependent activation had occurred.

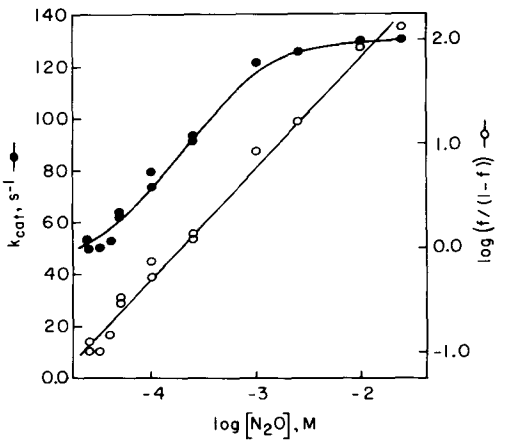

Fig. 1. Effect of N_2O concentration on the reaction rate of nitrous oxide reductase from *W. succinogenes*. The plot of k_{cat} vs. log[N_2O] (filled circles) is sigmoidal as expected for titration of a binding site. The linear log-log plot (open circles) involves the function f / (1 - f) which is defined in the text. The slope of the log-log line is 1.05.

represents a two-state Wyman function where f is the fraction of enzyme molecules in the activated state. The data define a straight line with slope of 1.05. The values of f are defined by Eq. 1 for which

$$f\,k'_{cat} + (1-f)\,k''_{cat} = k_{cat} \qquad 1$$

k'_{cat} = 130 s^{-1} from the plot of k_{cat} vs. log[N_2O] and k''_{cat} = 42 s^{-1}. This value of 42 s^{-1} was estimated by iteration, the criterion for which was that log(f / (1 - f)) vs. log[N_2O] should conform to a straight line without regard to slope.

The two state model can be understood from Eq. 2-4,

$$E + nN_2O \overset{K}{=} E(N_2O)_n \qquad 2$$

$$[E(N_2O)_n] / [E] = f/(1-f) = K\,[N_2O]^n \qquad 3$$

$$\log(f/(1-f)) = n \log[N_2O] + \log K \qquad 4$$

where K is the collective association constant and E and $E(N_2O)_n$ are the unactivated (but not inactive) and activated forms of the enzyme, respectively. From the slope of the log-log plot of Fig. 1, n = 1.05 and $K_d = 1/K$ = 130 μM. We believe that n may not be significantly different from 1 for technical reasons, in which case K_d = 200 μM. In either case, the results suggest a 1:1 complex between N_2O at an effector binding site.

The mass spectrometric M_r for the subunit of nitrous oxide reductase was estimated to be 95,540±39. The singly and doubly changed species that were observed were inferred to be $(M + H)^+$ and $(M + 2H + 1$ sinapinic acid$)^{2+}$. The M_r estimated by this method is somewhat greater than the value of 88,000 estimated by SDS-PAGE (1). The value of 95,500 was used in the data workup of Table 1 and Fig. 1.

DISCUSSION. The existence of an allosteric site for N_2O is puzzling, because N_2O is such an extraordinarily weak electrophile and nucleophile that it has virtually no affinity for the common

redox states of transition metals and does not add to or react with the functional groups of proteins. Furthermore, because N_2O has appreciable and similar solubilities in water and organic solvents (9), the free energy of its transfer from water to a hydrophobic binding site should be small. N_2O can form a relatively stable Ru complex, $[Ru(II)(NH_3)_5N_2O]^{2+}$ (10-12), but this is the rare exception and Ru is not a transition metal. In the case of nitrous oxide reductase, N_2O presumably can bind to some special Cu site which also activates N_2O for reduction. This site may be a binuclear center (13-16) referred to as center Z, but in any case the strong interaction between N_2O and the enzyme active site is probably different from the weak interactions between N_2O and common cuprous and cupric complexes.

Because of the unusual difficulty in coordinating N_2O, we suggest that a) the catalytic site (K_{mN2O} = 2.5 μM) and the newly inferred allosteric site (K_d = 130-200 μM) are basically the same site, the one located on one subunit of the dimer and the other on the second subunit, and b) the allosteric site can become catalytically functional, thus allowing an increase of about two in k_{cat} to be realized. Whether the two sites are permanently different or identical but alternating cannot be decided at present. This model implies that turnover-dependent activation of nitrous oxide reductase by N_2O may represent basically a switch from single to double site activity within the enzyme dimer.

The possibility that activation by N_2O involves a monomer to dimer conversion seems at the moment unlikely, in spite of the existence of monomeric forms of nitrous oxide reductase from *Rhodobacter* (f. gen.*Rhodopseudomonas) sphaeroides* (17) and *Achromobacter cycloclastes* (18). The enzyme from *W. succinogenes* is largely or entirely dimeric from gel filtration chromatography (1) at concentrations 20-40 times greater than those routinely used in the kinetic experiments. If the enzyme were significantly dissociated in the kinetic experiments and the monomer were inactive or of substantially lower activity than the dimer, one would expect the specific activity (or k_{cat}) not to be constant but to increase with increasing enzyme concentrations. For technical reasons related to the half-time for turnover-dependent inactivation, we can vary the enzyme concentration in kinetic studies over only a 5-fold range (80-400 ng x ml^{-1}), but within that range the specific activity appeared to be essentially constant.

ACKNOWLEDGMENT. This work was supported by Grant DCB 88-16273 from the National Science Foundation.

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