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THE DECOMPOSITION OF S-NITROSATED DITHIOLS: A MODEL FOR VICINAL NITROSOTHIOLS IN ENZYMES

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Abstract: Model dithiol compounds were S-nitrosated and the kinetics of their decompositions were studied. Rate constants for intramolecular homolysis of dinitrosated dithiols varied from 1.6 to 10 x 10^{-3} s^{-1}. Rate constants for intramolecular heterolysis of mononitrosated dithiols varied from 4 to 32 x 10^{-4} s^{-1}. Similar reactions may account for the effects of NO and nitrovasodilators on some enzymes. © 1997 Elsevier Science Ltd.

Nitric oxide (NO), nitrovasodilators like nitroglycerin, and many other NO-donor compounds are known to effect many physiological and pathophysiological processes.[1] Many enzymes are also effected by these compounds.[2] Relaxation of smooth muscles by NO and NO-donors is thought to be due to their activation of a large heterodimeric soluble guanylate cyclase (sGC) with approximately 30 thiol groups and one heme moiety.[3] Creatine kinase[4] and protein kinase C[5] are inactivated by S-nitrosothiols. Type I adenylyl cyclase[6] is no longer activated by calmodulin after treatment with NO-donors.

Activation of sGC by NO or NO-donors is usually attributed to the binding of NO to its heme moiety.[7] Oxidations of thiol groups, in some cases,[8] also stimulated sGC activity, however, without the involvement of NO or any NO-donor compounds. None of the other mentioned enzymes have a heme moiety but all have multiple thiol groups and may have one or more pairs of closely spaced or vicinal thiol groups. In all cases, the effects of NO-donors are reversed by treatment with dithiothreitol (DTT).

Under physiological conditions, S-nitrosothiols undergo rapid transnitrosation in the presence of thiols.[9] Both low molecular weight thiols and the thiol groups of proteins appear to undergo such reactions.[10] In cases of vicinal thiol groups in the enzymes mentioned above, an NO moiety might be transferred to either one or both of the thiol groups. It is important, therefore, to understand the properties of such derivatives, in particular, the nature and kinetics of the reactions which they undergo. In this report, we present results pertaining to the stability of the mono and dinitroso derivatives of DTT, DTT(NO), and $DTT(NO)_2$, and three other dithiols as models for what may transpire upon the S-nitrosation of vicinal thiol groups in proteins. These results suggest a mechanism whereby S-nitrosation might affect temporary changes in the activity of an enzyme by facilitating disulfide formation, and provide valuable information as to how DTT, and other dithiols, may promote NO formation from simple S-nitrosothiols and sodium nitroprusside.[11]

Experimental

S-nitrosation of dithiols: S-nitrosations were carried out by mixing thiols with sodium nitrite at pH 1.5 to 2 and room temperature.[10,12] Equimolar concentrations of glutathione and sodium nitrite were used to prepare *S*-nitrosoglutathione (GSNO). For dinitrosothiols (e.g., $DTT(NO)_2$), a slight excess of nitrite to dithiol was used (~2 to 5%). Mononitrosated dithiols (i.e., DTT(NO)) were prepared similarly but with at least a 20-fold excess of thiol (DTT: $NaNO_2$ >10:1). The reactions were followed, in each case, by monitoring their UV-visible absorptions with a Hewlett-Packard 8452 spectrophotometer As shown in Figure 1, both DTT(NO) and $DTT(NO)_2$ (0.25 mM) have characteristic S-nitrosothiol absorption peaks at ~334 nm and 544 nm (inset, 10 mM). Peak intensities of the former, ~950 $M^{-1}cm^{-1}$ at 334 nm and ~18 $M^{-1}cm^{-1}$ at 544 nm, were similar to those of GSNO and other mononitrosothiols and approximately half of those of $DTT(NO)_2$. The dinitrosothiols were not isolated in pure forms, however, S-nitrosations were complete as evidenced by HPLC analysis and by electrospray mass spectra of $DTT(NO)_2$ and $DTE(NO)_2$, *m/z* 230 ($M + NH_4^+$) and *m/z* 235 ($M + Na^+$) with a PE Sciex API 300 mass spectrometer.

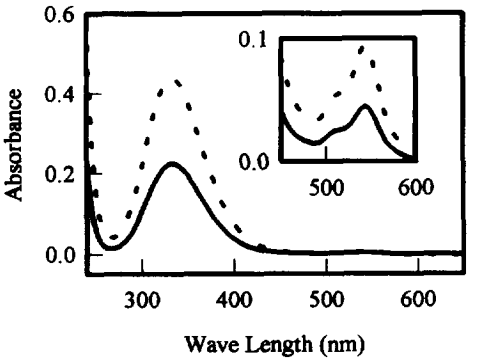

Figure 1. Spectra of DTT(NO) (—) and $DTT(NO)_2$ (- - -).

Kinetic studies: Decompositions of dinitrosothiols were followed by monitoring absorbance changes at 334 nm in 100 mM Tris-HCl buffer, 1 mM EDTA, at pH 7.4. First-order rate constants (k) were calculated from plots of $\ln(A/A_0)$ versus time. Decompositions of mononitroso derivatives were followed at a series of dithiol concentrations up to 5 mM in the same buffer and first-order rate constants (k_1) were obtained from the intercepts of plots of k_{obs} versus dithiol concentration. Decomposition of DTT(NO), prepared by transnitrosation with 0.1 mM GSNO in the presence of 1 to 5 mM DTT, was followed similarly in the same buffer.

Product analyses: Reaction products were analyzed by RP-HPLC on a C-18 column eluted with a linear gradient from 0 to 60% acetonitrile in 10 mM phosphoric acid at 1 mL/min for 30 min. Gaseous products of the reactions were determined with a Finnigan MAT-900 GC-MS spectrometer.

Results and Discussion

Decomposition of S-nitrosothiols by homolysis involves the generation of NO, thiyl radicals and finally disulfides. Rates are influenced by factors such as metal ions, light, pH, temperature, and the structure of the S-nitrosothiols.[12,13] Among these factors, metal ions have the most profound effects, particularly on *S*-nitroso compounds with an adjacent amino group.[12] After the elimination of free metal ions by including EDTA in the solutions, simple S-nitrosothiols are reasonably stable with half-lives ranging from 13.2 h to 46.5 h for S-nitrosocysteine[14] and GSNO,[12] respectively, and pH independent. It was suggested that the first step in the homolysis of S-nitrosothiols (Eq 1a) involves the formation of a thiyl radical followed by rate-determining dimerization of thiyl radicals to give the disulfide (Eq 1b).[15] Reactions between thiols and S-nitrosothiols are thought to proceed by nucleophilic attack of the thiol anion on the S-nitrosothiol to produce a disulfide and NO^- (Eq 2a). The latter combine to give N_2O as final product (Eq 2b). Rates of this heterolytic reaction are thiol concentration and pH dependent.

$$RSNO \rightleftharpoons NO + RS^{\bullet} \quad \text{(Eq 1a)} \qquad RSNO + RS^{-} \longrightarrow RSSR + NO^{-} \quad \text{(Eq 2a)}$$

$$2RS^{\bullet} \longrightarrow RSSR \quad \text{(Eq 1b)} \qquad 2NO^{-} + 2H^{+} \longrightarrow N_2O + H_2O \quad \text{(Eq 2b)}$$

$$\begin{matrix} SNO \\ SNO \end{matrix} \longrightarrow \begin{matrix} S \\ | \\ S \end{matrix} + 2NO \quad \text{(Eq 3)} \qquad \begin{matrix} SNO \\ S^{-} \end{matrix} \longrightarrow \begin{matrix} S \\ | \\ S \end{matrix} + NO^{-} \quad \text{(Eq 4)}$$

When the thiol in question is a dithiol, homolysis and heterolysis become intramolecular reactions and are expected to be faster. We have examined the mono and dinitrosated derivatives of four dithiol compounds, dithiothreitol (DTT), dithioerythritol (DTE), DL-6,8-thioctic acid (TA), and 2,3-dimercaptopropanol (DMP). Figure 2 shows typical time courses for the homolyses of $DTT(NO)_2$ and $DTE(NO)_2$. First-order rate constants, calculated from plots of $\ln(A/A_0)$ versus time as shown in the inset of Figure 2, are listed in Table 1. The decomposition of $DTT(NO)_2$, $DTE(NO)_2$, and $TA(NO)_2$ were 2 to 3 orders of magnitude faster than those of most monothiols. Half-lives thus were 6.4, 7.4, and 1.1 min, respectively, as compared to ~6 h for $DMP(NO)_2$ and 12.8 h for the S-nitroso derivative of β-mercaptoethanol (β-ME) under similar conditions.

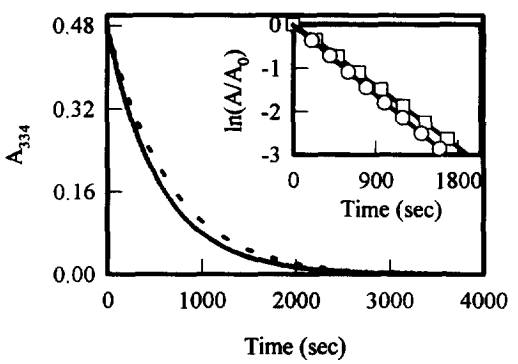

Figure 2. Homolysis of 0.25 mM $DTT(NO)_2$ (—) and $DTE(NO)_2$ (- - -). Inset: First-order plots for $DTT(NO)_2$ (O) and $DTE(NO)_2$ (□).

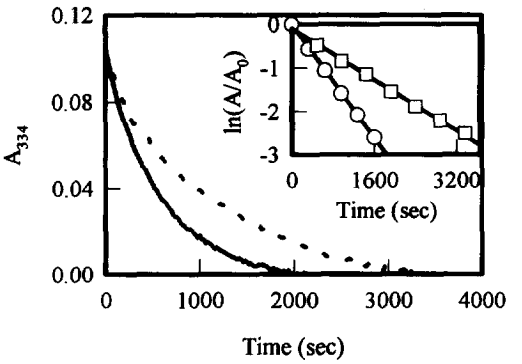

Figure 3. Heterolysis of 0.1 mM DTT(NO) (—) and DTE(NO) (- - -). Inset: First-order plots for DTT(NO) (O) and DTE(NO) (□).

Since a large excess of dithiol over $NaNO_2$ was required to obtain mononitrosothiol derivatives, the heterolysis of DTT(NO) and other mononitrosated dithiols were studied under two conditions. First, DTT(NO) and other mononitrosated dithiols were allowed to decay in the presence of various concentrations of free dithiols. Figure 3 shows time courses for two such reactions. Values of k_{obs}, calculated from the slopes of pseudo first-order plots (inset), were then plotted against the DTT or DTE concentration as shown in Figure 4. The rate equation for these heterolyses is shown as Eq 5. The intercepts of the lines in Figure 4 were the first-order rate constants (k_1) of intramolecular reaction of mononitrosated dithiols. The slopes were the second-order rate constants (k_2) for the intermolecular reactions of DTT(NO) and DTE(NO) with the corresponding dithiols. Values of k_1 and k_2 for all four

mononitrosated dithiols under these conditions are presented in Table 1. Data for the decomposition of S-nitroso-β-mercaptoethanol are also included for comparison. Heterolyses of mononitrosated dithiols were also studied using GSNO as a donor of NO. Since rates of transnitrosation are 2 to 3 orders of magnitude faster than those for heterolysis and a large excess of DTT over GSNO was used to ensure the completion of transnitrosation, values of k_{obs} should be governed by k_1 and k_2 (Eq 5). Values of k_1 and k_2 for DTT(NO) under these two conditions were in fact very similar as shown in Table 1. Half-lives for the intramolecular heterolysis of DTT(NO), DTE(NO), and TA(NO), calculated from k_1 , were 12.1, 28.9, and 3.6 minutes, respectively. In contrast to the homolysis of $DTT(NO)_2$ and other dinitrosothiols, these latter reactions increased rapidly with pH (Fig. 5) in accord with the nucleophilic mechanisms of Eqs 2a and 4.

Exceptionally slow intramolecular decomposition of both DMP(NO) and $DMP(NO)_2$, as indicated by an undetectable k_1 for heterolysis and very low k for homolysis, suggests formation of the cyclic oxidized form of DMP is very difficult. Intermolecular heterolysis of DMP(NO), however, was rapid, consistent with observation of polymeric reaction products, and nothing resembling a monomeric disulfide form of DMP, by RP-HPLC.

$$\text{rate} = k_1[\text{DTT(NO)}] + k_2[\text{DTT(NO)}][\text{DTT}] \quad \text{(Eq 5)}$$

Table 1. Decomposition rate constants for S-nitrosated mono and dithiols at pH 7.4.

Thiols	Homolysis	Heterolysis	
	$k \times 10^3$ (s^{-1})	$k_1 \times 10^3$ (s^{-1})	k_2 ($M^{-1}s^{-1}$)
DTT	1.8	0.9 [a]	0.78 [a]
		1.0 [b]	0.78 [b]
DTE	1.6	0.4 [a]	0.42 [a]
TA	10.0	3.2 [b]	20.2 [b]
DMP	0.032	n/d [c]	103.6 [b]
β-ME	0.015	n/d [c]	0.05
Cys [d]	0.015	n/d [c]	0.03

(a) For synthesized mononitrosated dithiols.
(b) For reactions of GSNO and dithiols.
(c) Not detectable.
(d) Data from ref 14.

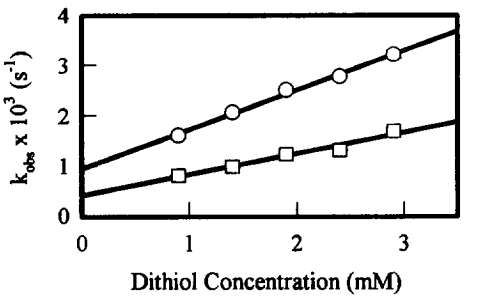

Figure 4. Heterolyses of DTT(NO) (O) and DTE(NO) (□) at different concentrations of DTT and DTE, respectively.

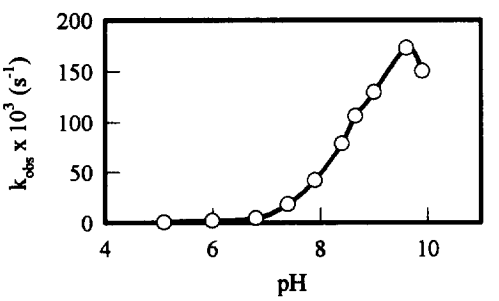

Figure 5. pH dependence for the heterolysis of DTT(NO).

In accord with their faster intramolecular rates, only cyclic disulfide forms were produced by homolysis or heterolysis of the other nitrosated dithiols as shown (e.g., Fig. 6). Under anaerobic conditions, NO (*m/z* 30) was the principle (>95%) gas product from the homolysis of $DTT(NO)_2$, whereas N_2O (*m/z* 44) was the main product (>95%) from the heterolysis of DTT(NO). Mixtures of DTT(NO) and $DTT(NO)_2$ gave both gas products and a 10-fold excess of GSNO over DTT was required for the efficient formation of NO.

Since our purpose was to study dithiols as models of vicinal thiol groups in proteins, we were particularly interested in the rates of intramolecular homo- and heterolysis. Those rates for the mono and dinitrosated derivatives of DTT, DTE, TA, and DMP, which all appear to be reasonable models for vicinal thiol groups in proteins, vary by more than three orders of magnitude due to differences in the distance between their respective thiol groups, their geometric and stereochemical relationships, their pKa values and their inherent reactivities. In all but the last case, proximity resulted in intramolecular rates two to three orders of magnitude greater than those observed in the case of simple mononitrosothiols. Half-lives for the intramolecular heterolysis of DTE(NO), DTT(NO) and TA(NO) ranged from 3 to 30 min and those for homolysis of the corresponding dinitroso derivatives ranged from 1 to 10 min. Changes in enzyme activities over similar periods of time following exposure to nitrovasodilators may be due to similarly rapid formation of disulfide bonds between vicinal thiol groups. The environments of two thiol groups and their spatial relationship to each other would determine the rate of such a process in the case of a specific enzyme and might serve as a timing device allowing for transient changes in activity over a predetermined period of time.

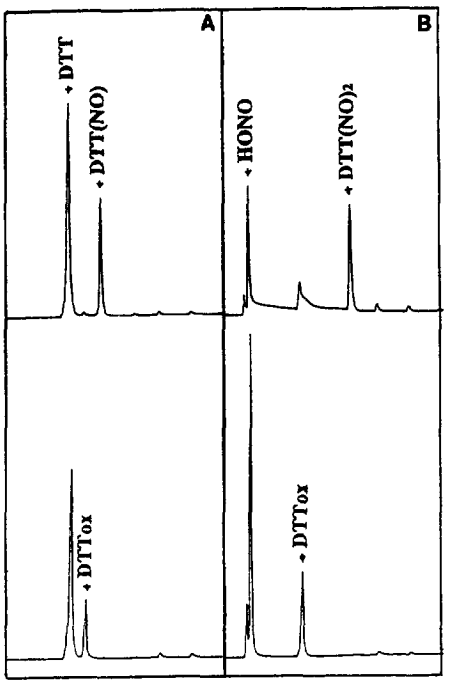

Figure 6. RP-HPLC profiles for the heterolysis of DTT(NO) (A) and the homolysis of $DTT(NO)_2$ (B) before (top) and after (bottom) reaction. HONO = nitrous acid.

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