

KINETIC ANALYSIS OF THE FORMATION OF NITROSYLMYOGLOBIN

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ABSTRACT

The formation of nitric oxide myoglobin (nitrosylmyoglobin) was followed in buffered solutions in which the concentrations of ascorbate, nitrite, chloride, myoglobin and hydrogen ion were varied systematically to determine their effect on the rate constants. The rate of formation of nitrosylmyoglobin was zero order with respect to the pigment. The orders for the other reactants were determined by plotting the zero order rate constants as functions of varying orders of each reagent to determine which order gave a linear plot. The results were used to develop a mechanism and a mathematical expression for the reaction. Two reaction sequences involving different nitrosating species were involved; 1, direct action of nitrous acid and 2, the formation of nitrosyl chloride. Both species then nitrosated ascorbate and ascorbic acid, by different mechanisms. The nitric oxide for nitrosylmyoglobin formation came from the nitrosated ascorbate.

Reference to a brand or firm name does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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INTRODUCTION

In a previous paper we reported results of the effect of the chloride ion on the formation of nitric oxide and nitrosylmyoglobin from ascorbate and nitrite at varying pH values (Sebranek and Fox 1991), and the effects of allowing the solutions to stand besides the presence of oxygen. In an earlier study of nitrosylmyoglobin formation in buffers with neither salt nor protein added (Fox and Thomson 1963) it was found that the observed kinetics could be accounted for by an expression derived from a series of reaction steps. The sequence included an unusual bimolecular reaction between two molecules of a mononitrosoascorbate intermediate to reform the initial reactants, which resulted in a half order dependence of the reaction rate on the ascorbate concentration ($r = k[A]^{1/2}$). Using the same mathematical techniques, and splitting the reaction data of the Sebranek and Fox (1991) study into their component parts, we incorporated the data from the initial phase of the reaction to determine the mechanism of the reaction.

EXPERIMENTAL

Order of the Dependences of the Reaction Rate on the Concentrations of the Reactants

The rate constants were plotted as functions of the concentrations of the various reagents to various powers, for example $[\text{Ascorbate}]^{1/2}$, $[\text{Nitrite}]^2$, etc. for powers of 0, $1/2$, 1 and 2. The process was repeated for each reactant until a straight line was obtained. Only the data from the initial phase of the reaction was used, before an equilibrium was reached. The concentration ranges studied were: Nitrite, 0.23–2.26 mM (15 to 150 ppm); ascorbate, 0.31–313 mM (55 to 550 ppm) and sodium chloride 0–0.7 M (0 to 4%). The concentration of the pigment was 0.05 mM.

In order to determine the rate constants for the chloride-catalyzed reaction, the rate constant for the reaction at each ascorbate/nitrite contribution with no chloride was subtracted from the rate constants for the same combination at each of the other of the chloride concentrations. These difference values were plotted as functions of varying powers of the reagent concentrations.

Terminology

According to IUPAC rules (CRC 1991) the nitric oxide metal complex and the cationic forms are to be called “nitrosyl” (Rule 7.323, Inorganic compounds) whereas the nitric oxide side chain bound to an organic compound is to be called “nitroso” (Rule A-2, Organic compounds). Interestingly enough this is a rever-

sal of the usual nomenclature for almost all other side chains have the “-yl” ending while complexes use the “-o” ending. For brevity we shall use the following abbreviations in the equations: AH_2 , ascorbic acid; $AHNO$, mononitrosoascorbic acid; AH , ascorbyl radical; $[N_T]$, molar concentration total nitrite; $[A_T]$, molar concentration total ascorbate; $[Cl_T]$, molar concentration total chloride; K_N and K_A , dissociation constants for nitric and ascorbic acids, respectively.

RESULTS

The plots where the regression was a straight line with a given power of the reactant concentration indicated the order of the reaction with respect to the reactant. Table 1 lists those orders for which the regressions were linear. As found earlier (Fox and Thompson 1963) the rate constants were zero order with respect to myoglobin, that is, no concentration dependence. The reaction with no chloride was half order with respect to ascorbate, as observed previously, but, except at the lowest concentration of ascorbate, the ascorbate concentration had no effect on the chloride-catalyzed reaction. The no-chloride reaction was half order with respect to the nitrite concentration but the chloride-catalyzed reaction was first

TABLE 1.
ORDER OF THE DEPENDENCE OF THE REACTION RATE ON THE CONCENTRATION OF
THE REACTANTS

Reactant	Order	
	Ascorbate/nitrite	Chloride-catalyzed
Myoglobin	Zero	Zero
Nitrite	Half	First
Ascorbate	Half	Zero ^a
Chloride	---	First
Hydrogen ion (pH)	First +	First +

^aAt ascorbate concentrations higher than 0.64 mM

order, clearly indicating two different nitrite mechanisms. The reaction curves were concave with hydrogen ion concentrations to the first power, convex with hydrogen ion concentrations to the second power, indicating a reaction somewhere between first and second order.

Myoglobin

The pigment remained in the native state and therefore was the mononitroso derivative. The rate of formation of nitrosylmyoglobin was constant over the course of the reaction, that is, zero order with respect to the pigment. Myoglobin was therefore complexing nitric oxide as fast as it was being formed, that is the formation of nitric oxide was the rate-controlling reaction. Since the other reagents were present in excess over that of the heme pigment, the total conversion of myoglobin to nitrosylmyoglobin involved decreases of only a few percent in their concentrations, hence the concentrations of the other reagents remained essentially constant during the reaction. Under these conditions the observed rate of formation was the sum and/or combination of the individual reaction rates forming nitric oxide and was dependent essentially on the initial concentrations of the reactants.

Ascorbate/Nitrite

The dependence of the reaction on the square roots of the ascorbate and nitrite concentrations (Table 1) is the result of a bimolecular reaction of an intermediate, produced by the two reagents, which yields the initial reagents. This is not the reverse of the forward reaction, but a different reaction mechanism (Fox and Thomson 1963; Fox and Ackerman 1968). In this reaction the postulated intermediate is mononitrosoascorbic acid (Dahn *et al.* 1960a).

Chloride

The rate constants obtained by subtracting the uncatalyzed reaction rates showed linear regressions with both nitrite and chloride concentration, that is, the reaction was first order with respect to these reagents. The rate dependence on ascorbate was neither first nor half order, principally because at ascorbate concentrations > 0.64 mM, the rate showed very little change with ascorbate concentration. ($\text{mM}^{-1} = 2.76 + 0.07 \text{ min}^{-1} \times [\text{Asc}]$, $R^2 = 0.053$, not significant). These observations preclude a chloride-catalyzed nitrosation of ascorbate to form the mononitrosoascorbic acid intermediate, since that would require that the rate of the chloride-catalyzed reaction be dependent on the square root of the concentrations of all three reagents. Ascorbate is necessary for the chloride-catalyzed reac-

tion as nitrite and chloride alone will not produce nitrosylmyoglobin. The chloride-catalyzed reaction mechanism therefore is different from the ascorbate/nitrite reaction mechanism, with the ascorbate reaction in both being fast but not rate determining in the chloride-catalyzed reaction.

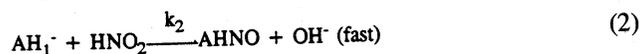
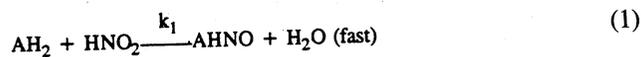
pH

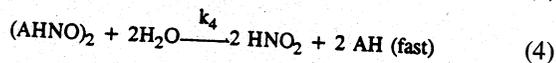
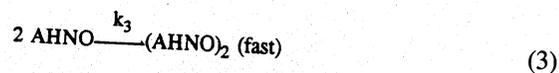
The hydrogen ion concentration dependence of the nitrite/ascorbate reaction was a function of the ionization of the two acids involved, ascorbic and nitrous. Since both of these acids have pK_a values near the pH range studied, the concentrations of either or both acids forms may be involved in the reaction. In this study, if only the acid forms of nitrite and ascorbate were used to calculate the rate of nitrosylmyoglobin formation, the rate values of pH 6.2 were too low, indicating an ascorbate/nitrite reaction involving an ionized form of one of the reactants. Dahn *et al.* (1960b) found the reduction of nitrous acid to be faster with the ascorbate ion than with ascorbic acid. In view of this finding and since the presence of the electron reduces the electrophilicity of the nitrite ion as compared with nitrous acid, we will assume that the reduction of nitrite by ascorbate takes place between nitrous acid and either the ascorbate ion or ascorbic acid.

The chloride-catalyzed portion of the reaction decreased with increasing pH to the point where the chloride concentration had no effect on the reaction rate at pH 6.2. Catalysis of nitrosation reactions by halogens is usually attributed to the formation of NOX (X = Cl, F, Br), (Fan and Tannenbaum 1973; Turney and Wright 1959; Stedman 1979), which are more powerful nitrosating species than nitrous acid. The reaction to form nitrosylchloride is between nitrous acid and the chloride ion (Stedman 1979; Turney and Wright 1959). Hence both the ascorbate/nitrite and the chloride catalyzed reactions required nitrous acid, but the chloride/nitrous acid reaction was too slow at pH 6.2 to be detected. It might be observed at this point that the concentrations of nitrous acid were very low at the pH values studied. The highest concentration of nitrite was 2.26 mM, which at pH 5.4 resulted in a concentration of 0.026 mM nitrous acid; at pH 6.2, 0.0022 mM.

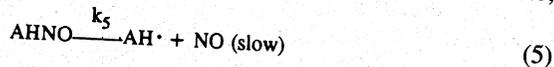
Reaction Mechanisms

In accordance with the dependencies shown in Table 1, the following reaction steps are written for the ascorbate/nitrite reaction:





The first four equations are written to account for the square root dependence on the ascorbate and nitrite concentrations (Fox and Thomson 1963), and involves the formation of a nitrosoascorbic acid dimer which reacts with water to form the original reactants. For the formation of nitric oxide from the intermediate, the reaction is:



The rate of formation and reaction of the intermediate AHNO is written:

$$\frac{d[\text{AHNO}]}{dt} = k_1[\text{HNO}_2][\text{AH}_2] + k_2[\text{HNO}_2][\text{AH}^-] - k_3[\text{AHNO}]^2 \quad (6)$$

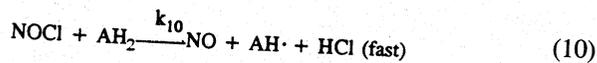
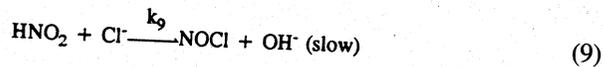
Reaction 5 is not included since it has been assumed to be slower than the first four reactions and therefore does not affect the equilibrium significantly. Making the steady-state assumption that the concentration of AHNO does not change during the course of the reaction, reaction 6 is set equal to zero. Rearranging the equation:

$$[\text{AHNO}] = \left\{ \frac{[\text{HNO}_2]}{k_3} (k_1[\text{AH}_2] + k_2[\text{AH}^-]) \right\}^{1/2} \quad (7)$$

Writing the rate expression for the formation of nitric oxide from the ascorbate/nitrite reaction and substituting the right hand side of equation 7 for the concentration of AHNO:

$$\frac{d[\text{NO}]}{dt} = k_5 \left\{ \frac{[\text{HNO}_2]}{k_3} (k_1[\text{AH}_2] + k_2[\text{AH}^-]) \right\}^{1/2} \quad (8)$$

For the chloride catalyzed reaction the equations for the formation of nitrosylchloride and nitric oxide are:



If reaction 9 is a slow reaction and reaction 10 fast, the formation of nitric oxide from the chloride-catalyzed reaction will be dependent only on the concentrations of nitrous acid and chloride but not ascorbate, except at low ascorbate concentrations, as was observed. The rate of formation of nitric oxide from the chloride-catalyzed reaction is dependent therefore only on reaction 9:

$$\frac{d[NO]}{dt} = k_9[HNO_2][Cl^-] \quad (11)$$

Since the reaction involved both acid and basic forms of the reactants, it is necessary to write the forms in terms of the total concentration of reactant added and the proton dissociation expressions and constants. When this is done for expressions 8 and 11 and the two combined to obtain the rate of formation of nitric oxide from both the ascorbate/nitrite and the chloride-catalyzed reactions, the following equation is obtained:

$$\frac{d[NO]}{dt} = k_5 \left[\frac{[N_T][A_T][H^+]}{k_3(K_N + [H^+])(K_A + [H^+])} \right]^{1/2} (k_1[H^+] + k_2K_A)^{1/2} + \frac{k_9[N_T][Cl_T][H^+]}{K_N + [H^+]} \quad (12)$$

Curve Fitting

This rate expression was fitted to the data using the NLIN procedure (Ralston and Jennrich 1978) in Release 6.03 (SAS 1987). For the calculation it was necessary to determine the values of only three constants, $K_1 = k_5(k_1/k_3)^{1/2}$, $K_2 = k_5(k_2/k_3)^{1/2}$ and k_9 , Table 2. While K_1 and K_2 are real values, without knowing the concentrations of the several intermediates the individual rate constants cannot be calculated. The calculated value of k_9 is the rate constant for the chloride-catalyzed reaction. A comparison of the calculated values with the experimental values for the various conditions is shown in Table 3. In order to visualize the shape of the curve needed to describe the rate of the reaction on the various reagents, a three-dimensional plot (response surface) is shown in Figure 1.

The asymptotic values of the three constants K_1 , K_2 and k_9 had coefficients of variation of 10.7, 9.7 and 15.9%, respectively. Since the pooled coefficient of variation determined from replicate experiments was 15.9%, the derived expression is as close a fit to the data as is possible. Another set of reaction steps might yield an expression which would give as good a fit, but not any better. As the number of reaction steps is the minimum required to account for the various dependencies, no simpler mechanism can be developed.

DISCUSSION

Most nitrosation research has been carried out in either dilute or strong acid solutions in which the nitrosating species are either the nitrous acidium ion H_2NO^+ or the anhydride N_2O_3 (Turney and Wright 1959; Austin 1961; Ridd 1961; Tedder 1970). The former requires a high hydrogen ion concentration while the latter requires a high concentration of nitrous acid, neither of which condition oc-

TABLE 2.

NON-LINEAR LEAST SQUARES SUMMARY STATISTICS DEPENDENT VARIABLE K			
Source	DF	Sum of Squares	Mean Square
Regression	3	0.00115179	0.0038393
Residual	132	0.00002246	0.0000017
Uncorrected total	135	0.00117425	
(Corrected total)	134	0.00040465	

Parameter	Estimate	Asymptotic Std. Error	Asymptotic Confidence interval	
			Lower	Upper
K ₁	0.00115707	0.00012348	0.00091282	0.00140133
K ₂	0.01887815	0.00182770	0.01526275	0.02249355
K ₉	0.00000554	0.00000088	0.00000380	0.00000728

Asymptotic correlation matrix of the parameters			
	K ₁	K ₂	K ₉
K ₁	1.000000	-0.486747	-0.819932
K ₂	-0.486747	1.000000	0.103038
K ₉	-0.819932	0.103038	1.000000

Equivalence of table two values with those of equation 12	
$K_1 = k_5 (k_1/k_3)^{1/2}$	$K_2 = k_5 (k_2/k_3)^{1/2}$
$k_9 = \text{mM}^{-1}\text{min}^{-1}$	

Dissociation values for ascorbate (A) and nitrite (N)	
$K_A = 6.76 \times 10^{-3}\text{mM}$	$K_N = 3.98 \times 10^{-1}\text{mM}$

curs in cured meats. Under the conditions of low nitrous acid concentration, the monomolecular nitrosation by nitrous acid becomes relatively more important than the bimolecular reactions forming the more reactive species. In a previous study, (Fox and Thomson 1963) the rate dependence was a fractional order with respect to nitrite but, following the conventional interpretation, the bimolecular formation of nitrogen trioxide was postulated as the initial step in the nitrosation reaction. In this study the dependence was clearly half order with respect to the

TABLE 3.
RATE CONSTANTS FOR THE FORMATION OF NITRIC OXIDE - AS MEASURED AND AS
CALCULATED BY EQUATION 12

mM NO ₂	mM Ascorbate	Percent NaCl					
		0%			4%		
		pH	Meas.	Calc	pH	Meas.	Calc
2.26	3.13	5.45	5.00	4.98	5.30	8.30	8.24
		6.32	1.46	1.41	6.19	1.66	1.92
2.26	0.64	5.47	1.65	2.21	5.30	5.50	4.65
		6.26	0.93	0.69	6.11	0.93	1.11
0.23	3.13	5.43	1.50	1.67	5.27	2.53	2.20
		6.26	0.55	0.49	6.11	0.41	0.62

nitrite concentration, which means that the initial reaction step was monomolecular in nitrous acid. The reaction mechanisms for the nitrosation of ascorbate were different for the nitrite/ascorbate and the chloride-catalyzed reactions, for in the former a semi-stable mononitrosoascorbate derivative was formed, with subsequent formation of nitric oxide, while in the latter the nitrosyl chloride was reduced directly and swiftly to nitric oxide. This is not an unusual phenomenon; for example, N₂O₃ nitrosated dibenzylamine or trimethylamine, but when NOCl was used the major products were the corresponding chlorides (Smith and Loeppky 1967). In our study, the product in both the nitrite/ascorbate and the chloride-catalyzed reactions had to have been nitric oxide, since the spectrum of the heme pigment was uniquely that of nitrosylmyoglobin. There was no direct nitrosation of the heme pigment by NOCl as ascorbate was required to form the nitric oxide pigment. The formation of nitric oxide as measured in this study occurred in the initial stages of the nitrite/ascorbate and chloride-catalyzed reactions and yielded no information as to subsequent reactions of the ascorbate. The reaction with nitrite does not produce measurable amounts of the ascorbyl radical (Fox *et al.* 1981), which means that either nitric oxide was formed without the formation of the radical or that the ascorbyl radical was very reactive with nitrite and was oxidized as fast as it was formed (Dahn *et al.* 1960b).

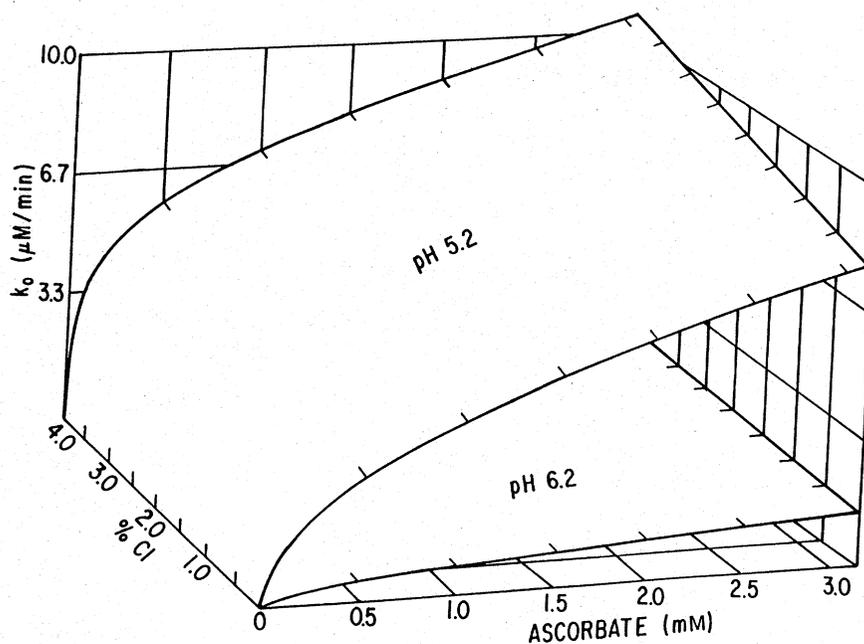


FIG. 1. THREE-DIMENSIONAL PLOT (RESPONSE SURFACE) FOR THE EFFECT OF CHLORIDE AND ASCORBATE ON THE RATE OF NITRIC OXIDE MYOGLOBIN FORMATION ($\mu\text{M}/\text{min}$) AT TWO DIFFERENT pH VALUES, 2.26 mM NITRITE

SUMMARY

Kinetic analysis of the reaction between nitrite/ascorbate and the catalysis by chloride has shown that the reaction proceeds by two separate mechanisms. The uncatalyzed reaction is the nitrosation of both ascorbic acid and ascorbate ion by nitrous acid, while the chloride-catalyzed reaction proceeds through nitrous acid to form nitrosylchloride, which subsequently is reduced by ascorbate. The mechanisms of the two nitrosation/reduction reactions differ in that the former produces a semi-stable mononitrosoascorbyl dimer, while the latter proceeds by a swift ascorbic acid reduction of nitrosylchloride.

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