FEBS Letters 345 (1994) 177–180

FEBS 14043

Kinetics and equilibria of *S*-nitrosothiol–thiol exchange between glutathione, cysteine, penicillamines and serum albumin

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Received 13 April 1994

Abstract

The kinetics and equilibria of *S*-nitrosothiol-thiol (SNO–SH) exchange reactions were determined using differential optical absorption. At pH 7.4 and 37°C, k_2 values ranged from 0.9 $M^{-1} \cdot s^{-1}$ for the reaction between *S*-nitroso-glutathione (GSNO) and *N*-acetyl-penicillamine, and up to 279 $M^{-1} \cdot s^{-1}$ for the exchange between *S*-nitroso-penicillamine (penSNO) and GSH. SNO–SH exchange involving GSH/GSNO and cysteine/cySNO was relatively rapid, k_2 approx. 80 $M^{-1} \cdot s^{-1}$ with an equilibrium constant slightly in favour of GSNO. GSNO was strongly favoured in equilibrium with penSNO, k_{eq} 0.0039. In the case of SNO–SH exchange between *S*-nitroso human serum albumin (albSNO) and GSH or cysteine k_2 values were 3.2 and 9.1 $M^{-1} \cdot s^{-1}$, respectively. The results show that the initial rate of SNO–SH exchange between physiological albSNO (7 μM) and venous plasma levels of GSH and cysteine is very slow, < 1%/min. On the other hand, if a nitrosothiol such as cySNO were to enter a cell, it would be rapidly converted to GSNO (43%/s).

Key words: Nitrosothiol; Nitric oxide; Thiol; Nitrosothiol exchange

1. Introduction

S-Nitrosothiols (RSNO) can elicit many of the various physiological regulatory functions attributed to nitric oxide/endothelium-derived relaxing factor, including smooth muscle relaxation [1–4], platelet deactivation [5,6], immunosupression [7] and neurotransmission [8]. RSNO occur in human venous plasma mainly as *S*-nitroso-albumin (7 μM) [9], and 0.3 μM GSNO has been measured in human bronchial lavage fluid [10]. However, the significance of endogenous RSNO is unclear. They may serve as a storage form of NO, intermediates in the excretion of NO as nitrite ion, or as specific physiological mediators. Regardless of the significance, if any, of endogenous RSNO, there appears to be considerable therapeutic potential for exogenous RSNO in areas such as the regulation of blood pressure, prevention of blood clotting, bronchodilation and smooth muscle relaxation for surgical procedures. Hence, various RSNO have been the subject of recent patents. However, these low molecular weight compounds are generally thought to undergo very rapid [14] or 'instantaneous' [15] SNO–SH exchange reactions, which complicates the attribution of physiological effects to a specific nitrosothiol [16], but a kinetic and equilibrium analysis of SNO–SH exchange involving the important biological thiols, GSH, cysteine, serum albumin and thiol drugs, is lacking. We have therefore determined the kinetic and equilibrium constants of these reactions to assess their potential significance in vivo.

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Abbreviations: RSNO, *S*-nitrosothiol; SNO–SH exchange, *S*-nitrosothiol-thiol exchange; GSH, glutathione; GSNO, *S*-nitroso-glutathione; cySNO, *S*-nitroso-cysteine; penSNO, *S*-nitroso-penicillamine; SNAP, *S*-nitroso-*N*-acetyl-penicillamine; albSNO, *S*-nitroso-human serum albumin.

2. Materials and methods

2.1. RSNO preparation

GSH (free acid), cysteine · HCl, penicillamine, *N*-acetyl-penicillamine, human serum albumin (crystallised), bathocuproine disulphonate, deferoxamine mesylate and *n*-butyl nitrite were obtained from Sigma-Aldrich (Poole, UK).

Non-protein *S*-nitrosothiols were prepared by mixing solutions of 40 mM thiol containing 10 μM bathocuproine-disulphonate (BCS) and 40 mM HCl at 0–4°C with 1/8 vol. of 400 mM cold $NaNo_2$. The reaction appeared complete within 6 min. 2 vols. of buffer A (100 mM NaCl, 10 μM BCS, 10 μM deferoxamine mesylate, 30 mM Na-phosphate, pH 7.40) was then added and the solution kept on ice.

Human serum albumin was dissolved in buffer A to 150 mg/ml and 10 mM dithiothreitol and 10 mM EDTA added. After 1 h at 20°C, dithiothreitol and EDTA were removed by passage through Sephadex G-25 (Pharmacia-LKB, Milton Keynes, UK) equilibrated in buffer A. Albumin concentration was determined from the UV spectrum ($\varepsilon_{278} = 3.89 \times 10^4$ $M^{-1} \cdot cm^{-1}$ [17]). S-Nitrosylation of the albumin thiol was then achieved by incubation with a 5-fold molar excess of *n*-butyl nitrite at 37°C until no further increase in A_{334} was seen (approx. 8 min). 0.98 mol albSNO was obtained per mol albumin using $\varepsilon_{334} = 870$ $M^{-1} \cdot cm^{-1}$ for albSNO.

mQ-grade water ($R \geq 18$ M$\Omega \cdot$cm) and tissue culture-grade plastic labware were used for all solutions.

2.2. Quantitation of S-nitrosothiol–thiol exchange

Absorption spectra of freshly prepared *S*-nitrosothiols were obtained between 300 and 650 nm using a Perkin Elmer Lambda 5 spectrophotometer. The absorption peak at 600 nm, characteristic of *S*-nitroso-penicillamines, was used to monitor SNO–SH exchange of a penicillamine/penSNO/SNAP with either GSH/GSNO or cysteine/cySNO ($\varepsilon_{600} = 10.2$ $M^{-1} \cdot cm^{-1}$). To confirm that SNO–SH exchange

SSDI 0014-5793(94)00429-Y

occurred, rather than merely decomposition of penSNO/SNAP, the reactions were also monitored at 541 nm where there is a concomitant increase due to GSNO/cySNO formation. SNO–SH exchange involving GSH and cysteine or albSNO and GSH was analysed using the lower molar absorptivity of CysNO/albSNO compared to GSNO at 334 nm ($\varepsilon = 90\ M^{-1} \cdot cm^{-1}$; D.J. Meyer, unpublished observations).

2.3. Analysis of kinetics and equilibria of RSNO–thiol exchange

2.3.1. Method 1. k_2 and K_{eq} were determined by reacting equimolar amounts of thiol and *S*-nitrosothiol. *S*-Nitrosothiol, 0.5–2.5 mM in buffer A, was placed in a fresh polystyrene cuvette (Boehringer-Mannheim, Lewes, Sussex) at 37°C and $A_{334\ nm}$ or $A_{600\ nm}$ was monitored for 2 min to obtain the slow decomposition rate. An equimolar amount of thiol was added, mixed and the exchange reaction monitored until equilibrium was reached. The thiol and *S*-nitrosothiol components were interchanged and the reaction repeated. Experiments were repeated at least once, and in the case of thiol and *S*-nitrosothiol of penicillamine and GSH the experiments were also repeated at different sets of reactant concentrations. The second order rate constant (k_2) for each reaction was obtained by plotting 1/[first RSNO or first thiol] vs. *t* and the equilibrium constant (K_{eq}) was calculated, both from the ratio of k_2 values for the forward and reverse reaction and directly from the equilibrium attained in each experiment.

2.3.2. Method 2. For the slower exchange reactions (those not involving penicillamine) k_2 values were also obtained from pseudo-first order rate constants obtained from the reaction of a 10- to 15- fold excess of thiol with *S*-nitrosothiol. This method was also used for exchange between albSNO and GSH which differed in their molar absorptivity at 334 nm.

2.3.3. Method 3. albSNO and cySNO showed similar molar absorptivity at 334 nm. Therefore, to monitor SNO–SH exchange, use was made of the great sensitivity of cySNO to copper-dependent decomposition (D.J. Meyer, unpublished observations). Thus albSNO in buffer A was mixed with an excess of cysteine in the presence of 20 μM $CuSO_4$ (10 μM excess over bathocuproine disulphate content of buffer A). The first order decay of A_{334} was then due to the transfer of nitrosyl- to cysteine.

3. Results and discussion

An example of SNO–SH exchange involving GSH and penSNO using method 1 is shown in Fig. 1. The reaction yields an average k_2 of 279 ± 33 $M^{-1} \cdot s^{-1}$ (S.E.M., $n = 8$),

Table 1
Kinetics and equilibria of *S*-nitrosothiol–thiol exchange reactions

RSNO	[RSNO] (mM)	Thiol	[Thiol] (mM)	k_2 ($M^{-1} \cdot s^{-1}$)	K_{eq}[a]	K_{eq}[b]
GSNO (A)	2.3	Pen (B)	2.3	1.25		
	2.3		2.3	0.80		
	2.3		2.3	1.20		
PenSNO (C)	0.7	GSH (D)	0.7	316		
	0.7		0.7	190		
	1.4		1.4	222		
	1.4		1.4	215		0.0014–0.0043
	1.4		1.4	330		
	2.3		2.3	317		
	2.3		2.3	460		
	2.3		2.3	180		
					0.0039	
GSNO (A)	2.3	NAP (B)	2.3	0.9		0.83
SNAP (C)	2.3	GSH (D)	2.3	1.1		0.83
	0.7		7.0	1.25[c]		
					0.77	
CySNO (A)	2.3	NAP (B)	2.3	1.3		0.40
SNAP (C)	2.3	Cysteine (D)	2.3	3.0		0.38
	0.7		7.0	3.2[c]		
					0.42	
GSNO (A)	0.6	Cysteine (B)	0.6	83		0.91
	0.4		4.0	60[c]		
CySNO (C)	0.6	GSH (D)	0.6	102		0.83
	0.4		4.0	74[c]		
					0.83	
albSNO	0.2	GSH	3.0		3.2	
albSNO	0.2	Cysteine	3.0		9.1[d]	

Equilibrium constants are calculated for the reaction:

$$A + B \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} C + D \text{ where } K = \frac{k_2}{k_{-2}} = \frac{[C][D]}{[A][B]}$$

k_2 and k_{-2} are second order rate constants and [A], [B], [C] and [D] are the concentrations of reactants and products at equilibrium.
[a] K_{eq} calculated from the ratio of average second order rate constants.
[b] K_{eq} derived from individual reaction end-point (method 1, section 2.3.1.).
[c] k_2 obtained from pseudo-first order rate constant (method 2, section 2.3.2.).
[d] k_2 obtained from pseudo-first order rate constant (method 3, section 2.3.3.).

and an equilibrium which is greatly in favour of GSNO (Table 1). No significant difference was observed between D- and L-penicillamines (not shown). The reaction of GSH or cysteine with SNAP is about 2 orders of magnitude slower, the equilibria also favouring GSNO or cySNO, respectively, but to a lesser extent than with penicillamine (Table 1). Penicillamine is used clinically as a copper-chelating drug to treat Wilson's disease [18]. The relatively unfavoured formation of penSNO may contribute to its lack of side effects.

SNO–SH exchange involving cysteine and GSH yielded k_2 values of about 80 $M^{-1} \cdot s^{-1}$ in each direction. This means that at the low concentrations of cysteine and GSH in plasma, SNO–SH exchange is calculated to be very slow. For example with 0.3 μM GSNO (as found in bronchiolar lavage fluid [10]), the initial rate of exchange with a human venous plasma level of cysteine of 9.2 μM [19] would be approx. 0.2 nM/s (4%/min). On the other hand, inside cells the high concentration of GSH may result in the rapid conversion of imported *S*-nitrosothiols to GSNO. For instance 0.3 μM cySNO would be converted to GSNO by 5 mM GSH at an initial rate of 0.13 μM/s (43%/s).

k_2 values were obtained for the reaction of the major known human plasma protein *S*-nitrosothiol (i.e. albSNO) with cysteine (e.g. Fig. 2) and with GSH (Table 1). The initial rate of SNO–SH exchange between human venous plasma levels of albSNO (7 μM [9]) and cysteine (9.2 μM [19]) would be 35 nM/min (0.51%/min), and with GSH (4.6 μM [19]) would be 6 nM/min (0.09%/min).

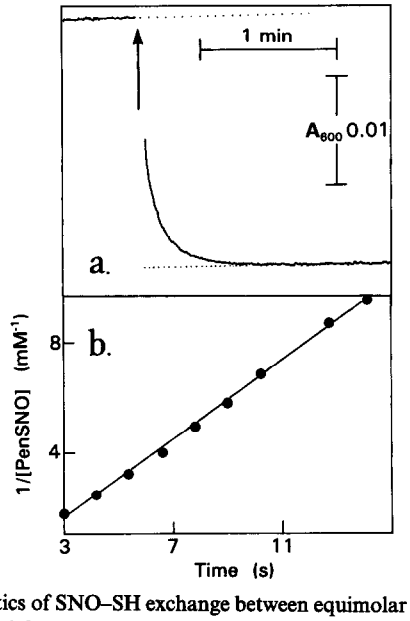

Fig. 1. Kinetics of SNO–SH exchange between equimolar penSNO and GSH. (a) To 2.3 mM penSNO in buffer A in a polystyrene cuvette was added (at arrow) 2.3 mM GSH and the SNO–SH exchange reaction monitored at 600 nm. (b) k_2 was determined from the slope of a plot of 1/[penSNO] (or 1/[GSH]) vs. time. K_{eq} was determined from the equilibrium concentrations of thiols and *S*-nitrosothiols (method 1, see section 2.3.1.).

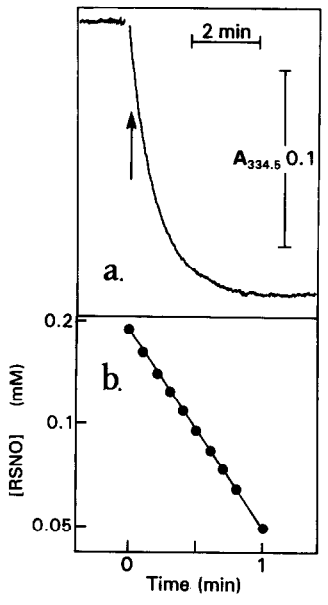

Fig. 2. Kinetics of SNO–SH exchange between albSNO and excess cysteine. (a) To 0.2 mM albSNO in buffer A plus 20 μM cupric sulphate in a polystyrene cuvette was added 3 mM cysteine. SNO–SH exchange was determined from the reduction in A_{334} due to the very rapid copper-dependent decomposition of cySNO. (b) A pseudo-first order rate constant was obtained from a plot of $\log_{10}$ [albSNO] vs. time, hence a value for k_2.

These rates are expected to be higher in arterial blood since the content of GSH and cysteine are higher, but are still remarkably low.

One may conclude that, in the extracellular milieu, where low molecular weight thiol concentrations are low, SNO–SH exchange is unimportant and potential *S*-nitroso-containing therapeutic agents should be relatively stable. Hence their physiological actions should be attributable directly to the applied agent. However, if the compounds were to enter or traverse cells (e.g. endothelium), conversion to GSNO is likely to be substantial and physiological effects are predicted to be due largely to GSNO.

Acknowledgements: This work was supported by the Cancer Research Campaign.

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