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PAPER

S-Nitrosocaptopril formation in aqueous acid and basic medium. A vasodilator and angiotensin converting enzyme inhibitor†

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The reaction of S-nitrosocaptopril (NOcap) formation was studied in both aqueous acid and basic medium. Captopril (cap) reacts rapidly with nitrous acid in strong acid medium to give the stable—in the timescale of the experiments—NOcap. The kinetic study of the reaction involving the use of stopped-flow, shows that at low sodium nitrite (nit) concentration, the reaction is first-order in both [nit], [H^+], and is strongly catalysed by Cl^- or Br^- (= X^-): rate = $(k_3 + k_4[X^-])[H^+][nit][cap]$. In aqueous buffered solution of acetic acid–acetate the reaction rate is much slower and the decomposition of NOcap was observed; however, the rate of NOcap decay is more than 30-fold slower than its formation. In aqueous basic medium of carbonate–hydrogen carbonate buffer, as well as in alkaline medium, the kinetics of the nitroso group (NO) transfer from *tert*-butyl nitrite (tBN) to cap was studied using either conventional or stopped flow methods. In mild basic medium, the NOcap decomposes. The NOcap formation is first-order in both tBN and cap concentrations, and the reaction rate increases with pH until to, approximately, pH 11.5, above which value it becomes pH independent or even invariable with the [OH^-]. Kinetic results show that the thiolate ion of cap is the reactive species. In fact, the presence of anionic micelles of sodium dodecyl sulfate (SDS) inhibits the reaction due to the separation of the reagents; whereas, cationic micelles of tetradecyltrimethylammonium bromide (TTABr) catalyse the reaction at low surfactant concentration due to reagents concentration in the small volume of the micelle. The rate equation is: rate = $k_f K_{SH}$[cap][tBN]/(K_{SH} + [H^+]). The rate of NOcap decomposition in mild basic medium is first-order in both [cap] and [NOcap], and decreases on increasing pH; but, in alkaline medium the NOcap is stable within the timescale of the experiments. Based on the results, the NOcap decomposition yields the disulfide compound that is formed in the nucleophilic attack of the –SH group of cap to the sulfur electrophilic center of NOcap, –S–N═O. The resulting rate equation is: rate = k_d[H^+][cap][NOcap]/(K_{SH} + [H^+]).

Introduction

Activation of guanylate cyclase and inhibition of angiotensin converting enzyme (ACE) are the two major mechanisms for vasodilation.[1,2] NO is a biological transmitter that mediates in vasodilation and in angiogenesis.[3–5] The interactions of NO with sulfhydryl-containing molecules and enzymes, the S-nitrosation reactions (also referred to as S-nitrosylation),[6–8] affect protein function and determine the many effects of NO *in vivo*. The nitrosation of the thiol group of captopril (cap) results in the formation of S-nitrosocaptopril (NOcap).[9,10]

The process does not alter the ability of cap to inhibit ACE and provides an additional mechanism by which NOcap can affect vascular relaxation. Both properties in the same molecule make it potentially a pharmacologically very interesting NO-donating drug.

It is of particular interest to optimize the various experimental conditions of NOcap formation, as well as to investigate its stability, since the decomposition of NOcap releases the biological significant species (NO and cap) to the *in vivo* system. In addition, S-nitrosothiols can also modify protein structure and function by inducing mixed disulfides and intramolecular disulfide in the direct attack of the thiol/thiolate group of enzyme-lysine to the S-nitrosothiol. In this sense, Wang et *al.*[11] studied the effect of S-nitrosothiols on the cellular regulatory mechanism of papain inactivation. Since many disease states such as muscular dystrophy, inflammation, and rheumatoid arthritis are associated with elevated proteolytic activity of cysteine proteases, much attention has been paid to the rational design and synthesis of selective inhibitors of these types of enzymes.[12,13] Papain is

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one of the most studied plant cysteine proteases that shares many features with physiologically important mammalian cysteine proteases. The active site of papain and others cysteine proteases (enzymes capable of hydrolysing peptide bonds), contain an essential cysteine sulfhydryl and histidine imidazole unit.[14] Wang's results suggest that inactivation of papain by S-nitrosothiols could be due to a direct attack of the reactive thiolate of cysteine unit (Cys^{25}) in the enzyme active site, on the sulfur of S-nitrosothiols to form a mixed disulfide between the inactivator and papain.[11]

The aim of this work is to explore the best experimental conditions of NOcap formation *in vitro* and its stability as a function of the acidity, with the goal of exploring the mechanism of enzyme inactivation by NOcap. For that, we investigated the NOcap formation in both acid and basic medium. In acid medium, sodium nitrite (nit) was used as the NO-precursor, whereas the NO-transfer from *tert*-butylnitrite (tBN) to cap has been applied in the formation of NOcap in basic medium. The reaction has also been studied in aqueous micellar solutions of both anionic micelles of sodium dodecyl sulfate (SDS) and of cationic micelles of tetradecyltrimethylammonium bromide (TTABr). Our results clearly support the hypothesis of Wang on the basis of a mechanism that yields disulfide bonds in the interaction of –SH and –SNO groups at pH values close to that of the imidazolium (pK_a~8.5)[14] of the active site of cysteine proteases. The results in micelles show the effective interaction of the cap anion with the cationic micelles of TTABr; this feature is consistent with the presence of imidazolium cation on the active site of cysteine proteases such as papain.

Experimental

Materials

Captopril or N-[(S)-3-mercapto-2-methyl propionyl)-L-proline of the higher available purity was obtained from Sigma and was used as received. *t*-Butylnitrite was synthesized by treating the corresponding alcohol with sodium nitrite in aqueous sulfuric acid, purified by fractional distillation under reduced pressure and stored at low temperature over molecular sieves to prevent hydrolysis.[15] All other reagents were of the maximum commercially available purity and were used as supplied.

Stock solution of *t*-butylnitrite was prepared daily in dioxane. A small volume of this solution (<100 μL) was dissolved in weakly basic medium (~2 mM carbonate buffer or sodium hydroxide) in which it is stable during the required time. The percentage of dioxane in the final reaction mixture never exceeded 1% by volume. The rest of the solutions were prepared with water that was firstly deionised and subsequently twice distilled (the first distillation over potassium permanganate).

The addition of a small concentration (20 μM) of EDTA (which would complex the possible trace quantities of metal ions such as Cu^{+2}/Cu^{+} that catalyse the S-nitroso compounds' decomposition) to the reaction sample did not affect the kinetic results.

Techniques

The pH was controlled using buffer solutions of acetic acid–acetate or carbonate–hydrogen carbonate and was measured with a Crison 2001 pH-meter equipped with a GK2401B combined glass electrode and calibrated using commercial buffers of pH 4.01, 7.02, and 9.26 (Crison). The reported [buffer] refers to the total buffer concentration. In strong acid ($HClO_4$) and alkaline (NaOH) media the acidity is given as $[H^+]$ or $[OH^-]$, respectively.

Kinetics of fast reactions were studied on a Bio-Logic SFM-20 stopped-flow system interfaced with a computer and operated by a Bio-Kine32 software (V4.51, 2009), noting the increasing absorbance at 325 nm due to the S-nitrosocaptopril.

The UV–vis spectra and kinetics of slow reactions ($t_{1/2}$>60 s) were recorded with a Kontron-Uvikon double beam spectrophotometer fitted with thermostatted multicell holders.

Methods

Experiments were performed under pseudo-first order conditions with either cap or nit as the limiting reagent concentration; experiments carried out in basic medium, with tBN being the NO-transfer, were performed with an excess of [cap] over [tBN]. Measurements of absorbance (A) *versus* time (t) fit perfectly the first-order integrated rate equation, $A_t = A_\infty + (A_o - A_\infty)\cdot\exp(-k_o t)$, unless otherwise indicated. The non-linear regression analysis of A–t data gives k_o, A_o, and A_∞ as optimizable parameters, with k_o being the pseudo-first order rate constant and A, A_o, and A_∞ being the absorbance values at times t, zero, and at the end of the reaction.

All experiments were carried out at 25 °C and the ionic strength has not been adjusted because the effect was negligible (at least at I <0.1 M).

Products characterization

Spectroscopic characteristics of reaction product coincide with those observed for NOcap that has been prepared and characterized by Loscalzo.[9] Decomposition of thionitrites, RSNO, in neutral aqueous solutions to give the corresponding disulfide and initially nitric oxide with quantitative yields is well documented in the literature.[6,16]

Results and discussion

1. Potentiometric titration

The captopril molecule is a mercapto-proline derivative that contains two acid ionisable groups: the carboxylic group of the proline residue and the thiol group of the propionyl moiety. Within the interval of acidity used in the present study, both acid groups of cap can be ionized. Because of that, the potentiometric titration has been performed from solutions that contain 8.8 mM of cap at ionic strength 0.10 M (NaCl) and titrating with a standardized sodium hydroxide solution in order to remove the two protons of the molecule and to obtain the macroscopic pK_{OH} and pK_{SH} (Scheme S1 of ESI†). Both equilibrium constants were calculated form the potentiometric titration data, which are shown in Fig. S1 of ESI,† by means of the Hyperquad 2003 program[17] as pK_{OH} = 3.52 ± 0.02 and pK_{SH} = 10.00 ± 0.01. The first value is attributed to the carboxylic group and the second one, to the thiol group. In this sense, the aqueous solution of 8.8 mM of cap at 0.10 M (NaCl) ionic strength has a pH = 2.82 in the absence of titrant; then, using the approach of Nyasulu *et al.*[18] that easily and rapidly determines K_a and K_b of uncharged substrates, one

calculates $pK_{OH} = 3.50$, which is in perfect agreement with the value calculated from the acid–base titration curve. For comparison purposes, the microscopic pK corresponding to the ionization of –COOH group in, for instance, cysteine varies from 2.00 to 4.99, whereas the microscopic pK of ionization of –SH group in the same compound varies between 7.44 and 9.87 and the pK_a of propane thiol is reported as 10.65.[19]

2. Characteristics of nitrosocaptopril formation reaction

Equimolar concentrations (14 mM) of cap and nit were mixed and allowed to react in strong acid medium ($HClO_4$ 0.060 M) at 25 °C. The reaction mixture turns red–brown instantaneously, due to the NOcap formation. The UV–visible spectrum, recorded between 200–650 nm, does not change with time, and shows strong absorption below 450 nm and a much less intense absorption band centered at 547 nm ($\varepsilon = 20\ M^{-1}cm^{-1}$). The longer absorption band is attributed to the forbidden $n_N \rightarrow \pi^*$ transition and determines the color of NOcap. In order to properly observe the band at lower wavelength, the solution should be diluted at least 5-fold; then, the characteristic band of S-nitroso compounds centered at 332 nm ($\varepsilon = 1020\ M^{-1}cm^{-1}$ for NOcap), attributed to the allowed $n_O \rightarrow \pi^*$ transition, is observed. There is a third strong absorption band below 260 nm, attributed to the $\pi \rightarrow \pi^*$, which cannot be used to follow the reaction due to the overlap with cap spectrum, whose aqueous solutions show strong absorption at wavelengths smaller than 250 nm, approximately, Fig. 1.

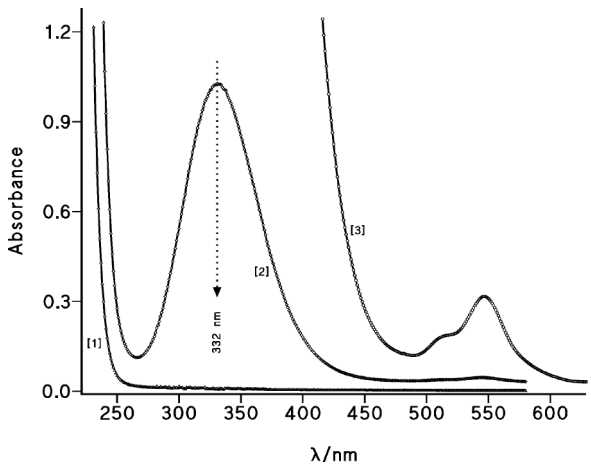


Fig. 1 UV–visible spectra of [1] cap 2.9 mM at $[H^+] = 0.05$ M; [2] NOcap formed in the reaction sample of [nit] = 1.0 mM, [cap] = 2.9 mM, and $[H^+] = 0.050$ M; and [3] NOcap formed in the reaction sample of [cap] = [nit] = 0.0 12 M and $[H^+] = 0.060$ M.

The NO-transfer to cap from S-nitroso-*N*-acetyl-D,L-penicillamie (SNAP) has been studied in aqueous basic medium.[10] The closeness of the maximum wavelength absorption of SNAP and NOcap and the resulting overlapping of the spectra complicates the kinetic study, which had been followed at 547 nm, using high reagent concentrations. The use of tBN as the NO-donor results in a non-complicated system from the spectral point of view.

When no excess nitrite is present in the reaction mixture, the generated NOcap is stable for longer. In this respect, the reaction followed during 15 h did not show appreciable decomposition, either in aqueous perchloric acid or acetic acid–acetate buffer (0.25–0.50 M, pH 3.6 to 5.2), or even in alkaline medium ($[OH^-] =$ 0.035 and 0.10 M).

To carry out the kinetic experiments, the concentration of the limiting reagent must not exceed 2 mM and the reaction progress was recorded at 325 nm. To check the reaction stoichiometry, the absorbance at 325 nm was measured at the end of the reaction for different [cap]/[nitrite] ratios; the results depicted in Fig. 2 show that the maximum value was obtained at equal concentration of both reagents, which indicates a 1 : 1 cap : nitrite stoichiometry in the nitrosation reaction.

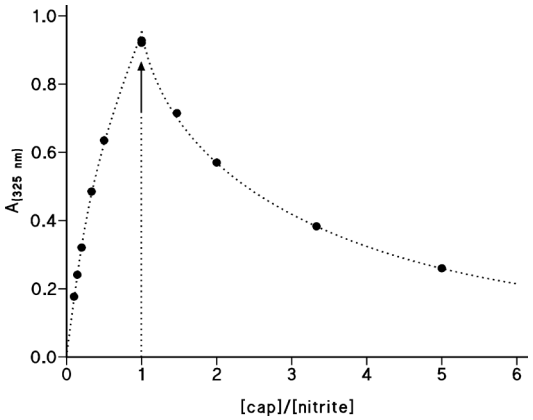


Fig. 2 Stoichiometric plot of the reaction of nit and cap in aqueous perchloric acid 0.050 M showing the variation of the absorbance readings at the end of the reaction for [cap] = [nit] = 2 mM mixed at different concentration ratios.

In strong acid medium, the cap-nitrosation reaction is too fast to follow it by conventional methods; therefore, the stopped-flow technique has been used. By contrast, in mild acid medium of acetic acid–acetate buffer the reaction was followed by conventional methods registering the increase absorbance also at 325 nm; Fig. 3(a) shows the reaction spectra in acetic acid–acetate buffer under conditions of [nit] < [cap]. The reaction spectra in aqueous basic medium of NOcap formation in the NO-transfer reaction from *tert*-butyl nitrite to cap are shown in Fig. 3(b). Notice the higher absorption of NOcap than either HNO_2/NO_2^- system or tBN, and the clear isosbestic point drawn at 277 nm in the reaction of cap + tBN → NOcap + *t*BOH (*t*-butanol).

3. NO-transfer in strong acid medium

The NOcap formation was studied in aqueous perchloric acid using the stopped-flow method. Several parameters that affect the reaction rate have been analysed including [nit], $[H^+]$, and $[X^-]$ (with $X^- = Cl^-$ or Br^-).

At constant $[H^+] = 0.025$ M the influence of [nit] was investigated in the range (3–30) mM with cap as the limiting reagent (0.7–0.07) mM. In every experiment the A versus t fits perfectly the first-order integrated rate equation with $\chi^2 < 0.002$. The pseudo-first order rate constant, k_o, increases with [nit] according to eqn (1), Fig. 4(a). The second-order term in [nit] will be significant at high nitrite concentrations. The non-linear regression analysis of k_o against [nit] to eqn (1) gives $k_1 = (56.1 \pm 0.8)\ mol^{-1}dm^3\ s^{-1}$ and $k_2 = (442.5 \pm 0.3)\ mol^{-2}\ dm^6\ s^{-1}$ (cc = 0.999_7).

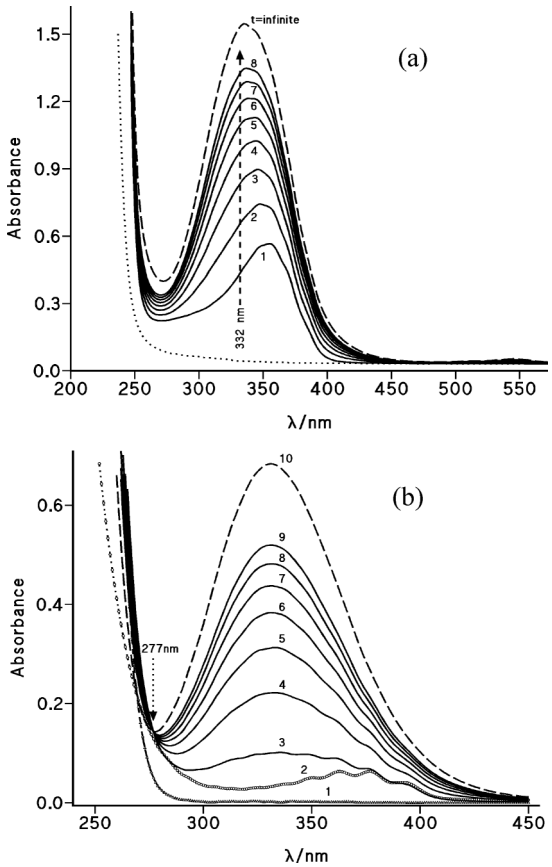


Fig. 3 (a) Reaction spectrum recorded at 5 min interval (scans 1 to 8) for the NO-transfer to cap 1.76 mM in aqueous acetic acid solutions ([buffer] = 0.55 M, pH 4.1) of [nit] = 1.0 mM; (···) no nitrite; (---) at the end of the reaction. (b) Reaction spectrum recorded at 2 min interval (scans 3 to 9) for the NO-transfer to cap 1.6 mM from tBN 1.1 mM in carbonate–hydrogen carbonate buffer ([buffer] = 0.066 M, pH 10.2); scans 1 and 2 show the spectrum of cap and tBN, respectively, under the same experimental conditions of the reaction, and scan 10 corresponds to the spectrum at the end of the reaction.

$$k_o = k_1[nit] + k_2[nit]^2 \quad (1)$$

Aqueous acid solutions of nit produce two nitrosating agents: the nitrosyl ion (NO^+) and dinitrogen trioxide (N_2O_3).[8,20] NO^+ is formed by the protonation and subsequent dehydration of nitrous acid ($HNO_2 + H^+ \rightleftarrows NO^+OH_2$, $K_{NO} = 3 \times 10^{-7}$ M^{-1}); the formation of N_2O_3 implies two nitrous acid molecules ($2HNO_2 \rightleftarrows N_2O_3$, $K_{N_2O_3} = 3 \times 10^{-3}$ M^{-1}); then, it is kinetically detected at high [nit]. Therefore, the first-order term in [nit] is due to cap nitrosation by NO^+ (NO^+OH_2), whilst the second-order term results from the nitrosation step by N_2O_3. Considering the relative values of k_1 and k_2, it can be concluded that at [nit] <~15 mM the nitrosation of cap goes mainly through NO^+, *i.e.* $k_1[nit] > 8.5k_2[nit]^2$. Therefore, in order to facilitate the quantitative treatment of the results, low [nit] was used in the following experiments.

The influence of $[H^+]$ was studied by working with either [cap] < [nit] or [cap] > [nit]. Under any of the two experimental situations, the A–t data fits perfectly the first-order integrated equation, *i.e.*, first-order with respect to [nit] in the first set of experiments and first-order with respect to [cap] in the second set of experiments.

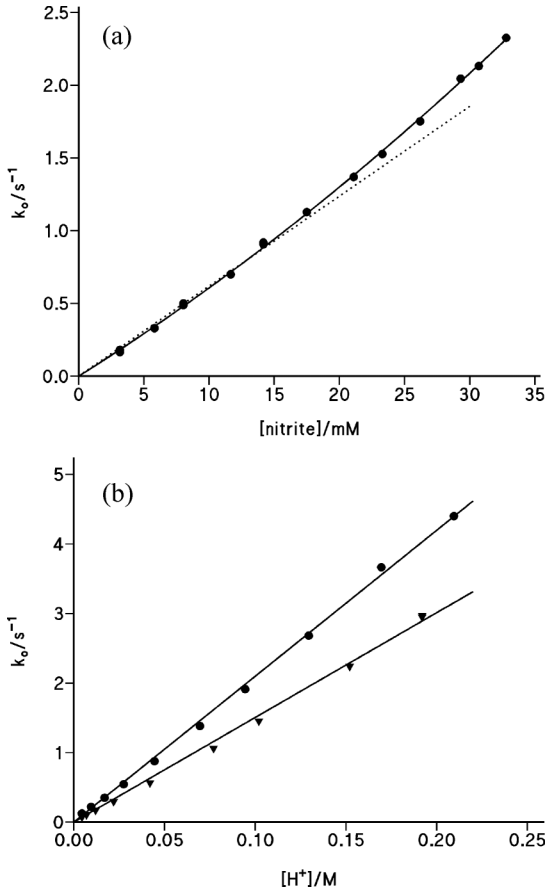


Fig. 4 (a) Variation of k_o as a function of [nit] for the reaction of NOcap formation in aqueous perchloric acid solutions at $[H^+] = 0.025$ M; (b) Plot of k_o against $[H^+]$ ($HClO_4$) obtained in the NOcap formation in aqueous solutions of (●) [cap] = 0.45 mM and [nit] = 8.5 mM and (▼) [cap] = 6.0 mM and [nit] = 0.45 mM, at 25 °C.

The variation of k_o as a function of $[H^+]$, depicted in Fig. 4(b), describes good straight lines with zero-intercept.

Under the experimental conditions of the results of Fig. 4(b), the contribution of the second-order term in [nit] of eqn (1) is much smaller than the first-order term (*i.e*, under the less favourable conditions of [nit] > [cap] it represents 6.7%), therefore, it can be stated that *rate* = $k_3[nit][cap][H^+]$. The least-squares fit of k_o *vs.* $[H^+]$ gives the slope value of (21.1 ± 0.2) mol^{-1} dm^3 s^{-1} when [nit] = 8.5 mM and of (15.1 ± 0.2) mol^{-1} dm^3 s^{-1} when [cap] = 6.0 mM. These results afford comparable values of k_3 = 2482 and 2517 mol^{-2} dm^6 s^{-1}, respectively, to the experiments performed with cap or nit as the limiting reagent.

The effect of Cl^- and Br^- was investigated at [cap] = 0.45 mM, [nit] = 9.0 mM and $[H^+] = 0.010$ M (for Cl^-) and $[H^+] = 0.014$ M (for Br^-). In either case, the reaction is strongly catalysed by Cl^- or Br^-, although the effect of bromide is higher than that of chloride. The plot of k_o *vs.* $[X^-]$ (X^- means Cl^- or Br^-) is a straight line with non-zero intercept, but, in order to highlight the different effect of both halide ions, Fig. 5 shows the variation of $k_o/([nit][H^+])$ as a function of $[X^-]$. The least-squares fit of the data yields the intercept (= k_3) and slope (= k_4) values of eqn (2) as k_3 = (2445 ± 50) mol^{-2} dm^6 s^1 and $k_4 = (44.3 \pm 0.5) \times 10^3$ mol^{-3} dm^9 s^{-1} for the experiments with Cl^- and k_3 = (2720 ± 70) mol^{-2} dm^6 s^1 and $k_4 = (155.6 \pm 0.9) \times 10^3$ mol^{-3} dm^9 s^{-1} for the experiments with Br^-.

Table 1 Experimental conditions used in the NOcap formation in aqueous perchloric acid from nitrous acid, values of the kinetic rate constants determined experimentally, k_i (i = 1,2,3, or 4), and their correspondence with the rate constants of the reaction mechanism of Scheme 1

[nit]/mM	[cap]/mM	[H⁺]/M	k_{exp}	equivalence	value	$K_{equilibrium}$	$k_{XNO}/M^{-1}\ s^{-1}$
3–30[a]	0.7–0.07	0.025	k_1	$k_{NO}K_{NO}[H^+]$	56.1 $M^{-1}\ s^{-1}$	$K_{NO} = 3 \times 10^{-7}\ M^{-1}$	$k_{NO} = 7.5 \times 10^9$
3–30[a]	variable	0.025	k_2	$k_{N_2O_3}K_{N_2O_3}$	442.5 $M^{-2}\ s^{-1}$	$K_{N_2O_3} = 3 \times 10^{-3}\ M^{-1}$	$k_{N_2O_3} = 1.5 \times 10^5$
8.5[a]	0.45	variable	k_3	$k_{NO}K_{NO}$	2482 $M^{-2}\ s^{-1}$	$K_{NO} = 3 \times 10^{-7}\ M^{-1}$	$k_{NO} = 8.5 \times 10^9$
0.45[a]	6.0	variable			2517 $M^{-2}\ s^{-1}$		$k_{NO} = 8.4 \times 10^9$
9.0[b]	0.45	0.10			2445 $M^{-2}\ s^{-1}$		$k_{NO} = 8.15 \times 10^9$
9.0[c]	0.45	0.14			2720 $M^{-2}\ s^{-1}$		$k_{NO} = 9.0 \times 10^9$
9.0[b]	0.45	0.10	k_4	$k_{ClNO}K_{ClNO}$	44.3 $M^{-3}\ s^{-1}$	$K_{ClNO} = 1.1 \times 10^{-3}\ M^{-2}$	$k_{ClNO} = 3.9 \times 10^7$
9.0[c]	0.45	0.14	k_4	$k_{BrNO}K_{BrNO}$	155.6 $M^{-3}\ s^{-1}$	$K_{BrNO} = 0.051\ M^{-2}$	$k_{BrNO} = 3.1 \times 10^6$

[a] No added Cl^- or Br^-; [b] variable [Cl^-]; [c] variable [Br^-]

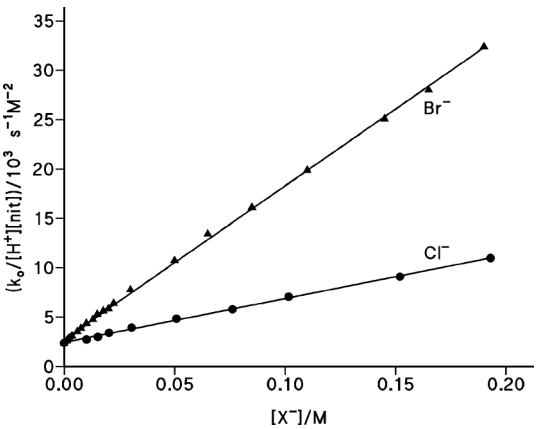


Fig. 5 Variation of k_o not dependent on [H⁺] and [nit]-*i.e.* $k_o/([H^+][nit])$- as a function of (●) [Cl^-] and (▲) [Br^-] obtained in the NOcap formation in aqueous perchloric acid solutions of sodium nitrite.

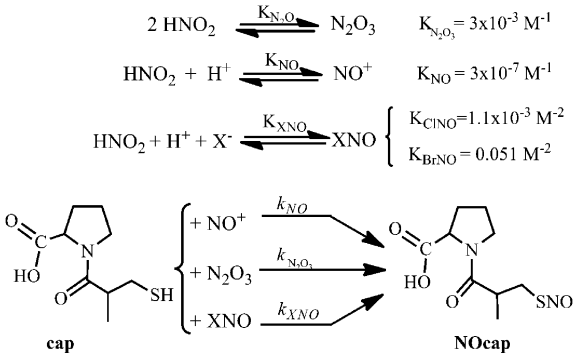
$$k_o = \left(k_3 + k_4\left[X^-\right]\right)\left[H^+\right][nit] \quad (2)$$

This rate equation suggests a reaction mechanism with two slow reaction steps due to nitrosation by both NO^+ and XNO. A common feature of many nitrosation reactions is the ability of halide ions and other nucleophiles to act as catalysts in acidic solution.[8,20] This is due to the equilibrium displacement towards the nitrosating agent NOX ($HNO_2 + H^+ + X^- \rightleftarrows XNO$, K_{XNO}), when compared with the equilibrium constant of NO^+ formation ($K_{NOX} = 1.1 \times 10^{-3}$ and $5.1 \times 10^{-2}\ M^{-2}$ for X = Cl and Br, respectively).

The rate constants for the nitroso group transfer from NOX follow the order NOCl > NOBr,[8,20,21] demonstrating that the catalytic effect is due to the thermodynamics of the formation of the nitrosating agent.

The overall observed experimental facts can be easily interpreted according to a reaction mechanism in which the thiol group of cap (but not thiolate) reacts in a rate limiting step with the nitrosating agent XNO, where $X^- = H_2O$, NO_2^-, Cl^- or Br^-. The corresponding reaction mechanism is shown in Scheme 1

In strong acid medium the stoichiometric [nit] is in the form of HNO_2, due to the pK_a = 3.14 at 25 °C of this weak acid.[22] Then, the rate eqn derived from Scheme 1 is that of eqn (3), which predicts each one of the observed experimental facts, of which, it is convenient to remark the fact that the straight line of k_o *vs.* [H⁺] plot with zero-intercept indicates that, firstly, the thiol group (and not the thiolate) is the reactive form of cap, and secondly, at the low



Scheme 1 Equilibrium and reaction steps that occur in the NOcap formation in aqueous acid solutions of sodium nitrite.

[nit] used in the experiments of Fig. 4(b), the second-order term on [nit] is negligible (the nitrosation step by N_2O_3 is distinguished experimentally by the absence of acid catalysis, apart from the second order dependence upon [HNO_2]).

$$\text{rate} = \left(k_{NO}K_{NO} + k_{XNO}K_{XNO}\left[X^-\right]\right)\left[H^+\right][nit][cap] + k_{N_2O_3}K_{N_2O_3}[nit]^2[cap] \quad (3)$$

The comparison of the experimental rate constants, k_i i = 1, . . . , 4, with the corresponding expression of eqn (3), allows the values of the bimolecular rate constants, k_{NO}, $k_{N_2O_3}$, and k_{XNO} reported in Table 1, along with the experimental conditions.

As is often found in nitrosation studies, the most reactive agent with thiol group of cap is the NO^+. The mean value of $k_{NO} = 8.3 \times 10^9\ mol^{-1}\ dm^3\ s^{-1}$ corresponds to a diffusion controlling rate constant. N_2O_3 is the less reactive species and ClNO transfers the nitrosyl group 10-fold faster than BrNO, due to the greater electronegativity of Cl than Br.

4. NO-transfer in mild acid medium

S-nitrosocaptopril formation has also been studied in aqueous solutions of acetic acid–acetate. Nitrous acid is a weak acid (pK_a = 3.15 at 25 °C).[22] Measurements at pH >~2.5 require the correction due to NO_2^- formation to be made. That is, the overall nitrite concentration is the sum of nitrite ion and nitrous acid; which implies that $[HNO_2] = [nit][H^+]/(K_a + [H^+])$. Therefore, the first observed feature is the reduction of the reaction rate as the pH increases, as can be see in Fig. 6(a). The presence of either Cl^- or Br^- enhances the reaction rate, due to the increase of the effective nitrosating agent, XNO, Fig. 6(b).

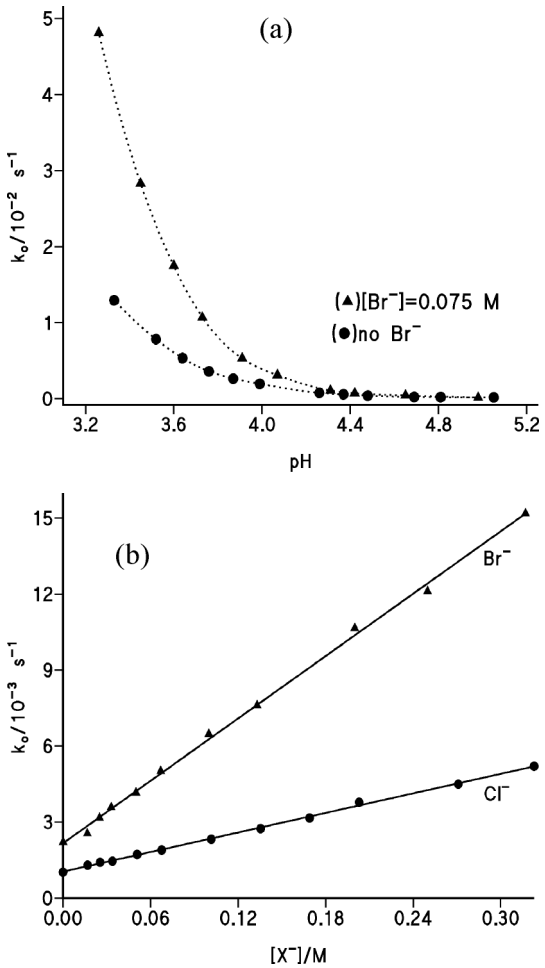


Fig. 6 (a) Effect of pH on k_o for NOcap formation in 0.31 M acetic acid–acetate buffer at [nit] = 12.5 mM, [cap] = 0.80 mM and (●) no added Br^- (▲) [Br^-] = 0.075 M; (b) variation of k_o for NOcap formation reaction in 0.25 M acetic acid–acetate buffer at [nit] = 12.5 M as a function of (●) [Cl^-] at pH 4.05 and of (▲) [Br^-] at pH 3.90.

The reaction rate is slightly affected by the total buffer concentration, as the data in Table 2 show, which may be attributed to the ionic strength effect. In this sense, at pH >4 more than 75% of the stoichiometric cap concentration is in the ionic form, *i.e.* the carboxylic group—pK_1 = 3.52—is ionized: $–COO^-$, and the nitrosating agent in the absence of Cl^- or Br^- is the NO^+.

Finally, as distinct from the reaction in strong acid medium, the NOcap is not stable under these experimental conditions. However, the decay rate is very slow, *e.g.* at pH 4.26 the observed rate constant has been measured as 4.1×10^{-5} s^{-1}, which is almost 20-fold lower than the rate of NOcap formation.

5. NO-transfer in basic medium

Aqueous basic solutions of nitrite salts do not generate nitrosating agents. One alternative is the use of alkyl nitrites, RONO, which are among the few nitrosating agents for basic media. In acid medium, alkyl nitrites such as tBN hydrolyse very fast to the parent alcohol and nitrous acid;[15] by contrast, their basic hydrolysis is a very slow process[23] and can be used as NO-donors under these experimental conditions[24,25] and in basic medium the nitrite ion is not a nitrosating agent. The NO-transfer to cap from S-nitroso-

Table 2 Values of the observed rate constant, k_o, measured in the NOcap formation in aqueous acetic acid–acetate solutions of nitrite as a function of total buffer concentration

[buffer]/M *pH 4.0*	$k_o/10^{-3}$ s^{-1}	[buffer]/M *pH 4.5*	$k_o/10^{-3}$ s^{-1}
$[cap]_o$ = 0.80 mM	[*nit*] = 10 mM	$[cap]_o$ = 0.80 mM	[*nit*] = 10 mM
0.15	1.41	0.10	0.364
0.20	1.42	0.15	0.377
0.25	1.45	0.20	0.381
0.30	1.51	0.25	0.391
0.45	1.59	0.30	0.410
0.60	1.65	0.45	0.435
0.70	1.77	0.55	0.452
0.80	1.78	0.65	0.467
0.95	1.79	0.70	0.475

N-acetyl-D,L-penicillamine (SNAP) has recently been studied in aqueous basic medium of phosphate buffer.[10] The closeness of the maximum wavelength absorption of SNAP and NOcap and the resulting overlapping of the spectra complicates the kinetic study, which had to be carried out in the visible spectral region by working with high reagent concentrations. The use of tBN as the NO-donor results in a very much simpler system, at least from the experimental point of view.

Therefore, in this study tBN was used in the NO-transfer reaction to cap. The reaction was studied in aqueous solutions of carbonate–bicarbonate buffer (pH range in between 9 to 11, approximately) and in alkaline medium (NaOH) by noting the increase in absorbance at 325 nm due to NOcap formation, and with tBN being the limiting reagent ([cap] ≥ 10[tBN]).

The absorbance–time (A–t) profiles suggest the existence of a rapid reaction (due to NOcap formation) followed by a much slower one (due to NOcap decomposition). The occurrence of both reactions is clearly observed at low pH (solutions of HCO_3^-/CO_3^{-2}); nevertheless, as the pH is increasing—*i.e.* in alkaline medium—only the reaction due to NOcap formation was observed. Fig. 7 shows representative A–t plots of both reactions.

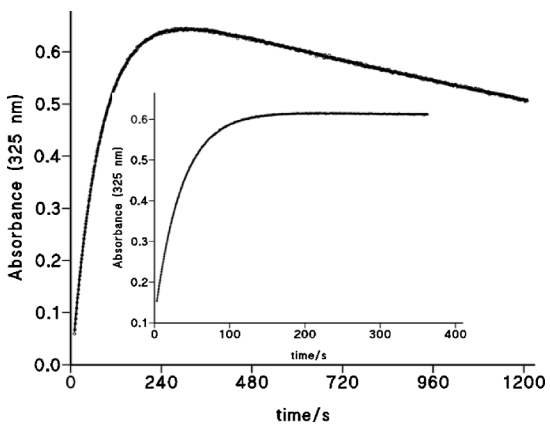


Fig. 7 Plot of absorbance readings at 325 nm against time for the reaction of cap 12.7mM and tBN 1.1 mM in aqueous basic medium of 0.085 M carbonate–bicarbonate buffer of pH 10.1, A–t data fits perfectly to $A = A_\infty + C_1 \cdot \exp(-k_1 t) + C_2 \cdot \exp(-k_2 t)$, the plot contains 1000 experimental points and the solid line (undistinguishable) of the fit process passes thorough every point; the inset shows the same plot for the reaction of 12.7 mM cap and 1.1 mM tBN in alkaline medium of [OH^-] = 0.14 M, but the A–t data fits perfectly to $A = A_\infty - (A_o - A_\infty) \cdot \exp(-k_1 t)$.

The influence of [cap] was analysed at pH 10.1, [buffer] = 0.085 M; NaOH solution was used to adjust the pH on increasing [cap] because of the ionization of the acid group of proline moiety and thiol. The A–t data profiles were fitted to the biexponential equation $A = A_\infty + C_1 \cdot \exp(-k_1 t) + C_2 \cdot \exp(-k_2 t)$ by non-linear regression analysis, with A_∞, C_1, k_1, C_2, and k_2 being the optimized parameters.[26,27] In order to obtain a proper convergence of the fitting process, the input values of C_1, and k_1 were estimated in a pre-fitting of only the increasing portion of the overall A–t curve, *i.e.* the reaction due to NOcap formation. The reported values correspond to that obtained in the fit of A–t data to the biexponential equation, with correlation coefficients >0.9999. Both rate constants, k_1 and k_2, increase proportionally to [cap], see Fig. 8, according to the equations $k_1 = (0.991 \pm 0.009)$ M^{-1} $s^{-1} \cdot [cap]$ and $k_2 = (2.70 \pm 0.03) \times 10^{-2}$ M^{-1} $s^{-1} \cdot [cap]$, but k_1 is more than 30-fold k_2.

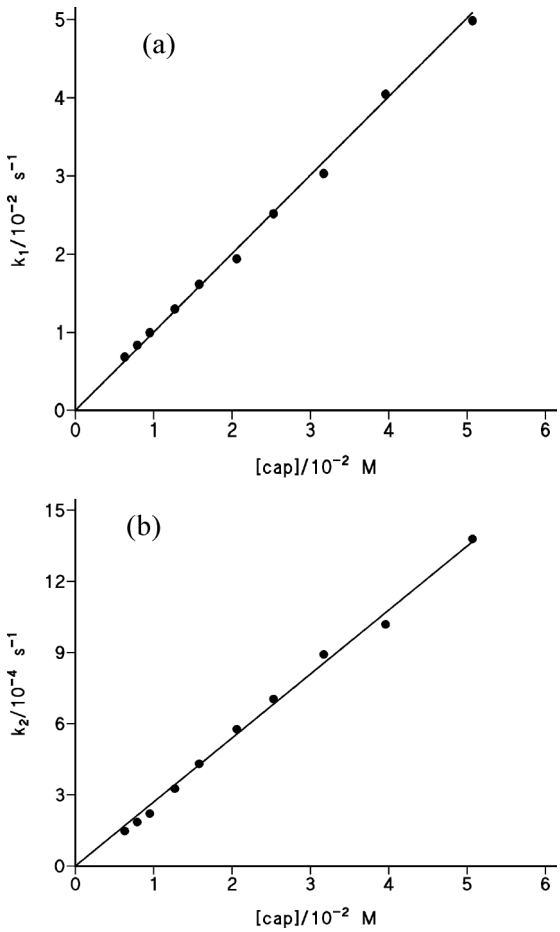


Fig. 8 (a) Variation of the pseudo-first order rate constant, k_1, as a function of [cap] for the NOcap formation, at [buffer] = 0.085 M of pH 10.1 (carbonate–bicarbonate buffer); (b) values of the rate constant of NOcap decomposition, k_2, measured as a function of [cap] in 0.085 M total [buffer] pH 10.1 (carbonate–bicarbonate buffer).

The pseudo-first order rate constant k_1 corresponds to the reaction between tBN and the thiol/thiolate group of cap, whereas k_2 is due to the decomposition reaction of NOcap and increases also with [cap], that is, the excess cap participates in the NOcap decomposition reaction.

To further elucidate the process, the effect of the pH was studied in aqueous solutions of 0.15 M carbonate–bicarbonate buffer and [cap] = 12.7 mM. At approximately pH <11, the A–t data profiles were fit to the double exponential equation, whilst above pH 11, or in alkaline medium, no decomposition of NOcap was detected, at least within the same time scale.

The k_1 values increase with pH by describing a sigmoid curve, Fig. 9(a). This experimental fact suggests the occurrence of an acid ionization equilibrium with the conjugate base being the reactive species; in other words, the ionization of the -SH group of cap to thiolate takes place and it is the nucleophilic attack of thiolate that provokes the NO-transfer. Scheme 2(a) illustrates the reaction steps.

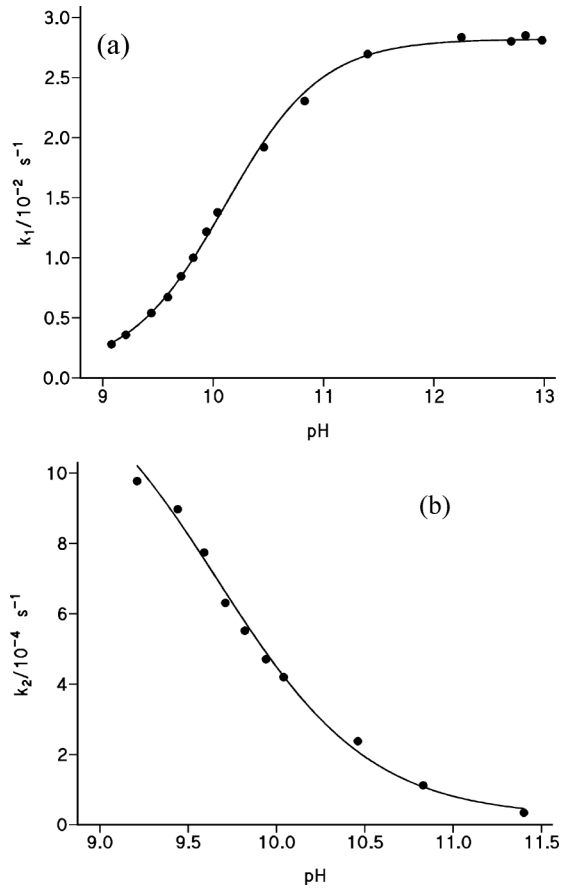


Fig. 9 Variation of the rate constants (a) k_1, determined in the NO-transfer from tBN to cap, and (b) k_2, determined for NOcap decomposition, as a function of pH; experimental conditions [cap] = 12.7 mM, [tBN] = 1.11 mM, [buffer] = 0.15 M (carbonate–bicarbonate), above pH~11.5 only NaOH has been used.

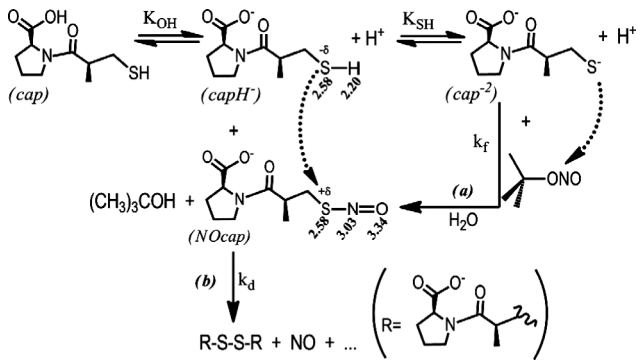
Taking into account that the stoichiometric cap concentration is $[cap]_o = [capH^-] + [cap^{-2}]$ and the acid ionization constant of thiol, K_{SH}, the eqn (4) can be obtained.

$$\text{rate} = k_1 [tBN] \quad \text{with} \quad k_1 = \frac{k_f K_{SH} [cap]}{K_{SH} + [H^+]} \qquad (4)$$

This eqn predicts that the graph of the reciprocal plot of k_1 against $[H^+]$ should result in a straight line, as experimentally was obtained, Fig. 10(a). Least-squares fitting of the data gives:

Table 3 Values of k_1 determined in the NO-transfer for tBN to cap as a function of [OH^-] and the limit value of the bimolecular rate constant k_f for the reaction of tBN and the thiolate group of cap, along with literature values[27] of k_f with different thiols

[OH^-]/M	$k_1/10^{-2}$ s^{-1}	k_f/M^{-1} s^{-1}	thiol	k_f/M^{-1} s^{-1}
0.073	2.67	2.10	limit value of k_f for tBN and the thiol[28]	
0.092	2.71	2.13	cysteine	1.7
0.110	2.85	2.24	cysteine-methyl ester	1.6
0.137	2.81	2.20	cysteine-ethyl-ester	1.5
0.165	2.65	2.10	*N*-acetyl-cysteine	1.8
0.202	2.66	2.10	glutathione	1.8
0.257	2.68	2.10	thioglycol acid	4.9



Scheme 2 Proposed equilibrium and reaction steps that occur in basic medium (a) in the NO-transfer reaction, k_f, from *tert*-butyl nitrite and captopril and (b) in the S-nitrosocaptopril decomposition, k_d, to give the disulfide compound in the reaction with the thiol group of captopril. The numbers on S, H, N, and O atoms refers to the corresponding electronegativity.

intercept = 38 ± 1 s and slope = (3.91 ± 0.045)×10^{11} M^{-1} s (cc = 0.999_2), from which values can be determined pK_{SH} = 10.01 and k_f = 2.07 M^{-1} s^{-1} for the bimolecular reaction between tBN and the thiolate group of cap. The kinetic pK_{SH} matches the value determined by potentiometry and also compares quite well with the published value,[10] pK_{SH} = 9.80. On the other hand, the value of k_f is in good agreement with either the values obtained in the nitrosation of other thiols by *tert*-butylnitrite,[28] see Table 3, or with the limit value directly determined in alkaline medium, that is, in conditions of no pH effect, and reported also in the Table 3.

By contrast, the observed rate constant of NOcap decomposition, k_2, decreases as the pH increases, and, at pH >11 no decomposition was observed; meanwhile, the k_1 reaches its maximum value and remains invariable on increasing [OH^-] as can be seen from data in Table 3. On the other hand, k_2 increases with [cap] at pH 10.1. These experimental facts suggest a decay reaction of NOcap with the participation of the thiol group of a second cap molecule, to give the disulfide compound, Scheme 2(b). Considering the electronegativity values of S, H, N, and O atoms (shown in Scheme 2), the S-atom of the thiol group is a nucleophilic center capable of attack on the electrophilic S-atom of thionitrite group to give the disulfide compound, RSSR.

The rate eqn will be *rate* = k_d[capH$^-$][NOcap], with k_2 = k_d[capH$^-$]. Considering that [cap] = [capH$^-$] + [cap^{-2}] and the acid ionization equilibrium of thiol group, K_{SH}, one easily arrives to eqn (5) that relates the experimental k_2 with the stoichiometric cap concentration and [H^+].

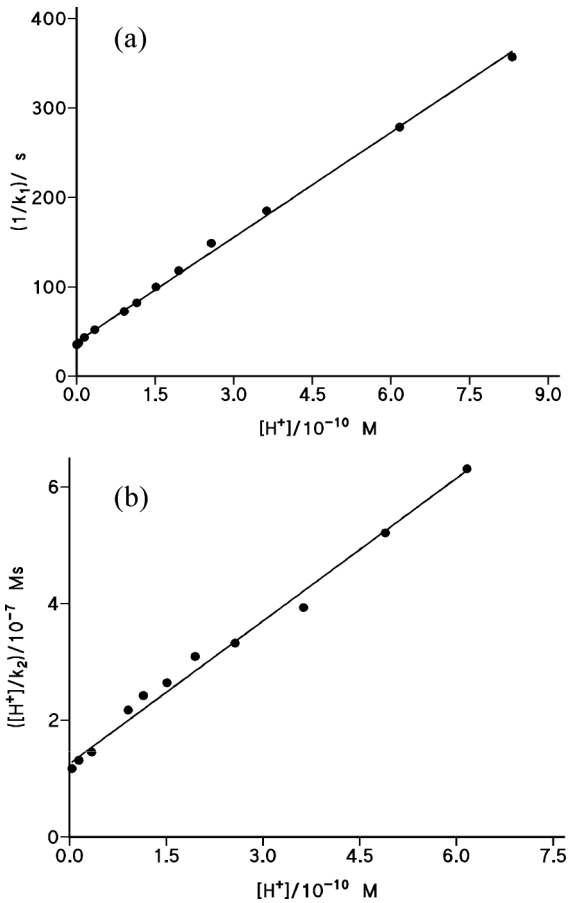


Fig. 10 (a) Reciprocal plot of k_1 against [H^+] to show the linear behaviour according to eqn (4); (b) linear-correlation of [H^+]/k_2 against [H^+] that predicts eqn (5).

$$k_2 = \frac{k_d[H^+][cap]}{K_{SH} + [H^+]} \quad (5)$$

According to this equation the plot of [H^+]/k_2 *versus* [H^+] should result in a straight line, as can be see in Fig. 10(b); in addition, the ratio of intercept/slope of the line equals K_{SH}. Least-squares fit of the linear plot gave: intercept = $K_{SH}(k_d[cap])^{-1} = (1.28 \pm 0.06) \times 10^{-7}$ M s and slope = $(k_d[cap])^{-1} = (8.15 \pm 0.25) \times 10^2$ s. From the quotient of both values one determines pK_{SH} = 9.8, in good agreement with the published value and the experimentally determined pK_{SH}, and k_d = 0.097 mol^{-1} dm^3 s^{-1}.

To further test our proposal of the NO-transfer from tBN to cap-thiolate group, the reaction has been carried out in aqueous

micellar solutions of both the cationic surfactant tetradecyltrimethylammonium bromide (TTABr) and the anionic surfactant sodium dodecylsulfate (SDS). The kinetics have been followed with the stopped-flow equipment at $[OH^-] = 0.065$ M and [cap] = 0.014 M and [tBN] = 1.2 mM. The experimental data of A–t fits perfectly the first-order integrate rate equation with $\chi^2 < 0.001$. The variation of the observed rate constant, *i.e.* k_1/s^{-1}, with the surfactant concentration can be seen in Fig. 11 for the case of either SDS (a) or TTABr (b).

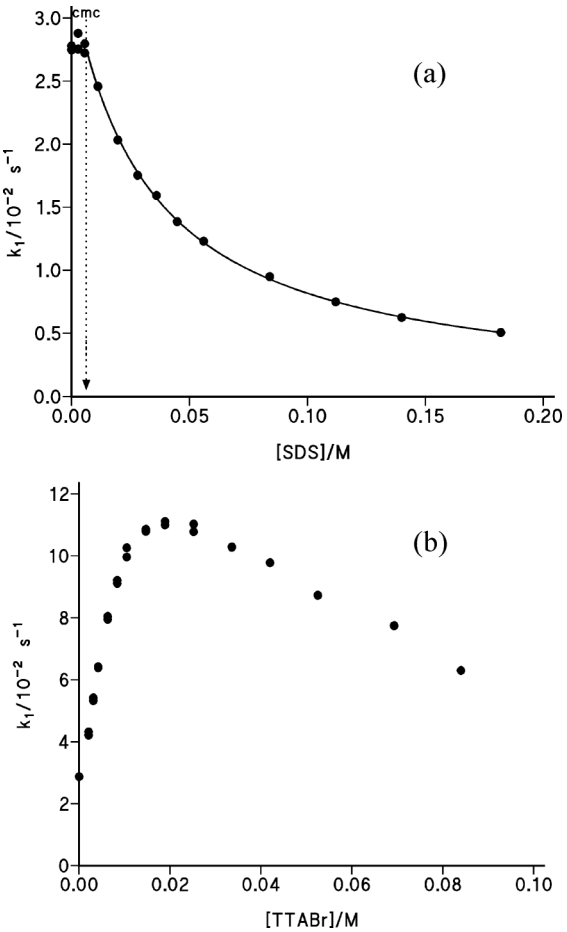
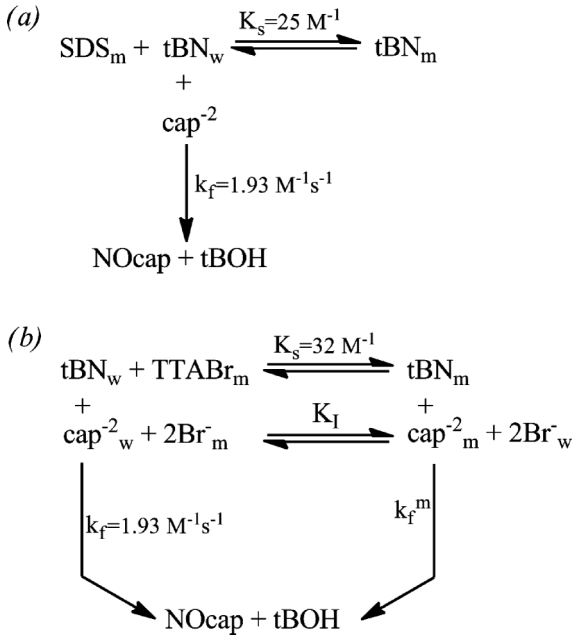


Fig. 11 Influence of (a) SDS micelles and (b) TTABr micelles on the rate constant k_1 measured in the NO-transfer from tBN to cap in alkaline medium, $[OH^-] = 0.065$ M, at [cap] = 14.3 mM and [tBN] = 1.2 mM. The solid line fits eqn (6); for parameters, see text.

It can be noted that anionic micelles of SDS inhibit the reaction throughout all the concentration range, whereas cationic micelles strongly catalyse the reaction at [TTABr] <0.020 M, approximately; a further increase of surfactant concentration results in a decrease of k_1 values. The observed k_1–[TTABr] profile is typically found when reactive anions (captopril thiolate ions in the present case) exchange with the surfactant counterions (Br^- in the present case).

t-Butylnitrite is a hydrophobic neutral compound that binds specifically to either cationic or anionic micelles.[29] The magnitude of the interaction depends on the micelle hydrophobicity, *i.e.* on the number of C-atoms in the hydrocarbon-chain of the surfactant. For the present case, previous studies in this lab have determined the binding constants of tBN as $K_s = 15$ and 32 M^{-1} respectively to SDS and TTABr micelles. Under the experimental conditions used, all cap is in the form of thiolate ions, *i.e.* cap^{-2} (Scheme 2). Therefore, the inhibition observed in the presence of SDS micelles is due to the reactants' separation: tBN associates to SDS micelles, whilst cap^{-2} is excluded from the micellar interphase by electrostatic repulsions, Scheme 3; therefore, only reaction in the water pseudophase is possible.



Scheme 3 Equilibrium and reaction steps that occur in the NO-transfer reaction form *tert*-butyl nitrite (tBN) to captopril (cap) in alkaline medium in the presence of (a) SDS micelles and of (b) TTABr micelles.

Considering that total tBN concentration is the sum of the concentrations in both aqueous and micellar pseudophases, $[tBN] = [tBN]_w + [tBN]_m$, and the distribution equilibrium constant, K_s, the eqn (6) is derived. In this eqn, $k_1{}^w$ is the pseudo-first order rate constant in the water pseudophase, and as defined previously, k_f is the bimolecular rate constant between tBN and thiolate ions of cap, and cmc represents the critical micellar concentration of SDS (~6.5 mM in the present conditions).[29,30]

$$k_1 = \frac{k_1^w}{1 + K_s\left([SDS]\text{-cmc}\right)} \text{ with } k_1^w = k_f\,[cap] \qquad (6)$$

The non-linear regression analysis of experimental k_1 – [SDS] data yields: $k_f = 1.93$ M^{-1} s^{-1} ($k_1{}^w = (2.74 \pm 0.02) \times 10^{-2}$ s^{-1}) and $K_s = (25.0 \pm 0.4)$ M^{-1} taking cmc = 6.5 mM. The k_f value agrees perfectly with those directly determined and listed in Table 3. The K_s value is higher than that determined in the acid hydrolysis of tBN;[29] however, the concordance of the results is acceptable if one takes into account that, firstly, in the published datum the sample solutions contain 1% dioxane and secondly, the presence of H^+ in the micellar interphase modifies its polarity.

By contrast, cationic micelles of TTABr catalyse the NO transfer reaction due to reagent concentration in the small volume of the micelles: tBN binds to TTABr micelles and cap^{-2} ions exchange with the micellar counterions, Br^-, according to the equilibrium:

$cap^{-2}{}_w + 2Br^-{}_m \rightleftarrows cap^{-2}{}_m + 2Br^-{}_w$, K_I. The quantitative treatment of the data requires the knowledge of K_I. In any case, the possibility of reaction in both water and micellar interphases, having similar reactivities, explains the observed increase in k_1 at low surfactant concentration (high $[cap^{-2}]_m$ due to low $[Br^-]$) and the subsequent decreasing as the Br^- ions, introduced with the surfactant, displace the cap^{-2} from the micellar interphase in the exchange equilibrium process, Scheme 3(b).

Conclusions

S-nitrosocaptopril is really formed in aqueous acid solutions of sodium nitrite or in aqueous basic solutions of *t*-butylnitrite. In either strong acid or alkaline medium the NOcap is stable, whereas in mild acid or basic medium it decomposes in the timescale of the experiments. The thiol is the reactive group towards the nitrosating agents for the acid medium, XNO ($X^- = H_2O$, NO_2^-, Cl^-, Br^-, ...), whereas the nucleophilic attack of the thiolate ion to the nitroso group of tBN provokes the transnitrosation reaction in basic medium. In this sense, the thionitrites' (RSNO) behaviour is quite different from that of alkyl nitrites (RONO). The lower electronegativity of sulfur atoms than that of oxygen (or nitrogen) atoms makes the S-atom of RSNO an electrophilic center. Then, the RSNO decomposes to the disulfide compound; whereas alkyl nitrites do not give the peroxide derivative. The present results showed that NOcap decomposition occurs under pH conditions similar to the imidazolium pK_a of the active site of cysteine proteases to yield the disulfide compound (RSSR) in the attack of the thiol group of a cap molecule to the thionitrite group of NOcap. Extrapolating to the *in vivo* system, if the thiol group would correspond to a cysteine molecule in, *e.g.* papaine, the RSSR formation changes the protein structure. This fact affects the protein function and, at the same time, the NO releases affect vascular relaxation. The results in cationic micelles justified the cationic character of the active site of cysteine proteases. In summary, the present results are in line with many papers proposing that the biological effects of nitrosothiols are due to their tendency to liberate NO or to modify other functional cysteine containing proteins through S-transnitrosation.

Acknowledgements

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