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Dynamic studies of transnitrosation of thiols of biological importance by the nitrosated 4,4′,4″,4‴-tetrasulfophthalocyaninecobaltate(III) anion in aqueous solution

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ABSTRACT

The kinetics of interaction of Co(III)TSPcNO (TSPc = 4,4′,4″,4‴-tetrasulfophthalocyanine) with various thiols of biological relevance, e.g., reduced glutathione (GSH), captopril (CapSH), *N*-acetyl-*L*-cysteine (NALC), and *L*-cysteine ethyl ester (LCEE) have been investigated spectrophotometrically. The observed rate constants for transnitrosation are all first-order with respect to the respective thiols. The second-order rate constants which were determined at physiological temperature, 37 °C are 258 ± 8, 159 ± 3, 66.7 ± 1.3 and 37.4 ± 0.6 $M^{-1}\,s^{-1}$, respectively. The second-order rate constants decreased according to the sequence LCEE > CapSH > GSH > NALC. The activation parameters ($\Delta H^{\neq}$ and $\Delta S^{\neq}$) were derived from the Eyring's equation. The experimental activation parameters were then correlated by an isokinetic plot, for the reduction of $[Co(III)TSPc(NO^{-})]^{4-}$ by the thiols, making use of the expression: $\Delta H^{\ddagger} = \Delta G_0^{\ddagger} + \beta_0 \Delta S^{\ddagger}$ where $\Delta G_0^{\ddagger}$ is the intrinsic free energy of activation, and β_o the isokinetic temperature. The plot which showed very good linearity ($R^2 = 0.997$), gave values of $\Delta G_o^{\ddagger}$ ($61 \pm 1\,J\,K^{-1}\,mol^{-1}$) from the intercept, and β_o (260 ± 11 K) from the slope. It is concluded that a common mechanism is adhered to in the reduction of Co(III)TSPcNO, irrespective of the type of thiol being used, to give the corresponding *S*-nitrosothiol, which is further confirmed by high performance liquid chromatography with mass spectrometric detector.

1. Introduction

Reactions leading to transnitrosation, i.e., transfer of NO in biological systems have generated considerable interest in recent years because of the important role of S-nitrosothiols in bioregulatory processes [1]. Biological thiols are reactive and ubiquitous, existing as products of sulfur metabolism. They possess similar chemical properties as alcohols (R-OH) in terms of pK, redox potentials, and the ability to form free-radicals [2]. These properties within the –SH group account for the uniqueness of thiol chemistry [2].

Thiol groups in general are required for the activity of many biologically important proteins, and are important in functioning as reducing agents and cellular antioxidants [3]. Whilst reduced glutathione is the principal thiol and redox buffer in mammalian cells and serum albumin [3], *L*-cysteine [4] exists predominantly as the extracellular low-molecular weight thiol.

Thiols are known to scavenge reactive oxygen species [5], and in particular have been shown to have anti-platelet effects at millimolar concentration levels [6]. Of more critical importance, is the ability of thiols to form S-nitrosothiols (RSNOs). The easiest and most convenient way to synthesize RSNOs is to nitrosate thiols via sodium nitrite under acidic conditions Eq. (1) [7].

$$RSH + HNO_2 \overset{H^+}{\rightleftharpoons} RSNO + H_2O \quad (1)$$

The generation of RSNOs also comes about via the S-transnitrosation of thiols [8].

$$RSNO + R'SH \rightleftharpoons R'SNO + RSH \quad (2)$$

The products of such reactions Eq. (2) are known to be detected at submolar levels in plasma and broncho-alveolar lavage fluid, presumably formed from the S-nitrosation of thiols, and cysteine residues of proteins [8]. The occurrence of transnitrosation has been confirmed by characterizing the disulfides formed as end products, as shown in Eq. (3) [7,9].

$$RSNO + R'SH \longrightarrow RSSR + RSS'R + R'SSR' \quad (3)$$

Principal targets of NO in bioregulatory systems are not only heme and non-heme iron centers, but also other transition metal centers such as Co (III). The proposition is made, that in cellular systems, metal–NO adducts play important roles in the nitrosating of various nucleophiles [10].

The investigation of the interaction of thiols with CoTSPcNO was undertaken in order to establish a mechanism of transfer of NO from the nitrosated product of CoTSPc.

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2. Experimental

Co(II)TSPc was prepared and purified according to the method established by Weber and Busch [11]. Solutions of the nitrosated complex were prepared *in situ* similarly to the preparation of NO solutions. NO gas was passed for approximately 3 h through 50 mL of a 5 mM solution of CoTSPc containing H_2EDTA^{2-} (100 μM). NO was generated by adding a saturated solution of $NaNO_2$ to 200 mL of a 0.2 M solution of HCl. All solutions used were deaerated by passing $N_2(g)$ through the closed system for approximately 45 min. The NO produced was passed through a 5 M solution of KOH to ensure the removal of higher oxides of nitrogen [12,13].

The NO stretching frequency obtained from infrared analysis of an aqueous solution was 1639 cm^{-1} which compares well with literature [12] (lit: 1500 to 1900 cm^{-1}), indicating the presence of the NO^- moiety and hence a cobalt(III) center [2,14].

Solutions of thiols (captopril, *N*-acetyl-*L*-cysteine, *N*-acetyl-*D*,*L*-penicillamine and *L*-cysteine ethyl ester) were freshly prepared before each spectrophotometric run, by dissolving the solid in H_2EDTA^{2-} solutions, and making up to the mark on the volumetric flask with deionized water. There were instances where stock solutions were prepared, from which required amounts were pipetted into volumetric flasks containing H_2EDTA^{2-} solutions. Solutions were used as soon as possible to minimize aerial oxidation.

2.1. Reaction stoichiometry

In a spectrophotometric titration, the reductants (thiols) and complex (Co(III)TSPcNO) were reacted at different ratios, with the complex concentration being held constant. This study was carried out in deaerated water at fixed temperature, and monitored at 669 nm. The absorbances recorded at the end of the particular reactions were plotted against the [reductant]/[complex] ratios to determine the number of moles of reductant reacting with each mole of nitroso-compound, as indicated by the breakpoint in the graphs plotted.

2.2. Kinetic measurements

The reactions were monitored spectrophotometrically at 669 nm, where the largest absorbance change occurred. This was carried out on a Hewlett Packard 8453 Diode Array spectrophotometer fitted with a Hi-Tech Scientific SFA-20 Rapid Kinetics Accessory (RKA) unit. All experimental solutions involving kinetic and thermodynamic measurements were thermostatted (±0.1 °C), using a RM6 Lauda Brinkmann thermostatted water bath. Solution pairs (same pH) of thiol and Co(III)TSPcNO were placed in the respective syringes on the RKA unit. The concentration of solutions was twice the desired final concentration and contained Na_2H_2EDTA (50 or 100 μM) to sequester traces of the contaminating metal ions (example Cu^{2+}) that may catalyse the reaction.

All kinetic experiments were carried out under pseudo-first-order conditions, with the thiol in at least 10-fold excess. Deionized water was used to make up all solutions. Values of pseudo-first order rate constants (k_{obs}) were obtained from the absorbance–time traces, which were fitted to Eq. (4) where A_o, A_∞ and A_t, are the absorbance values at initial, final and varying times, respectively.

$$A_t = A_\infty + (A_o - A_\infty)exp(-k_{obs}.t) \quad (4)$$

2.3. Product determination

In NO release reactions, the nitrite anion is known to be a by-product, and is determined via the standard diazotization reaction procedure [15]. Standard solutions (5–20 μM nitrite) were used to prepare a calibration curve according to literature. 0.5 mL of sulphanilamide solution (0.5 g sulphanilamide dissolved in 100 mL of 20% v/v hydrochloric acid) was added to 25.0 mL of a neutral nitrite solution. 0.5 mL of N-(l-naphthyl)-ethylenediamine dihydrochloride solution (0.3 g of the solid reagent dissolved in 100 mL of 1% v/v hydrochloric acid) was added to each flask. Absorbance readings were recorded at 541 nm on a HP 8453 Diode Array spectrophotometer after 10 min had elapsed. A blank solution was used to zero the instrument.

The experimental samples were treated similarly and the calibration curve used to calculate the concentration of nitrite present in solution.

Under acidic conditions, the nitrite ions cause diazotization of sulphanilamide (4-aminobenzenesulphonamide) to occur, and the end product is coupled with N-(1-naphthyl)-ehtylenediamine dihydrochloride) [16,17]. The intensity of the reddish coloured solution is directly proportional to the concentration of the nitrite anion, and is quantified spectrophotometrically.

2.4. Identification of S-nitrosocaptopril: LC/DAD/MS measurements

The confirmation of the formation of S-nitrosocaptopril was done by LC/DAD/MS, using an Agilent 1100 Series LC coupled to an Agilent 1100 Series MS Detector. DAD: 332 ± 10, ref. 360 ± 100. MSD: SIM mode, − ve ESI; 245; frag. 70 V; gain 40 V. MSD: SCAN mode, 200–300 range, frag. 80 V, gain 60 V. Pump: 0.5 mL/min, 95% water: 5% methanol, run time = 10 min. Column: 25 °C, Zorbax XDB-C18, 15 cm, 4.6 mm i.d., 5 mm particle size. Injection volume: 25 mL.

3. Results and discussion

3.1. Nature of the reaction

On mixing the colourless solution of the thiols, GSH, CapSH, NALC and LCEE, with the blue–green solution of Co(III)TSPcNO, a pale blue solution of the product formed within minutes. This is accompanied by a decrease in absorbance at 669 nm, and a corresponding increase at 300 nm. The repetitive scan representing the reaction of CapSH with Co(III)TSPcNO (Fig. 1) shows three isosbestic points at 288, 339 nm and 640 nm. Repetitive scans for the other thiols are shown in the supplementary data (Figs. S1, S2 and S3).

As displayed in Fig. 1 (a–b), the decrease in absorbance at 669 nm is accompanied by a blue shift from 669 to 661 nm. An equilibrium mixture of the monomer/dimer system of Co(II)TSPc is known to absorb in the region of 659 nm [12]. This strongly suggests the loss of NO from the complex with concerted reduction of the metal center to Co(II).

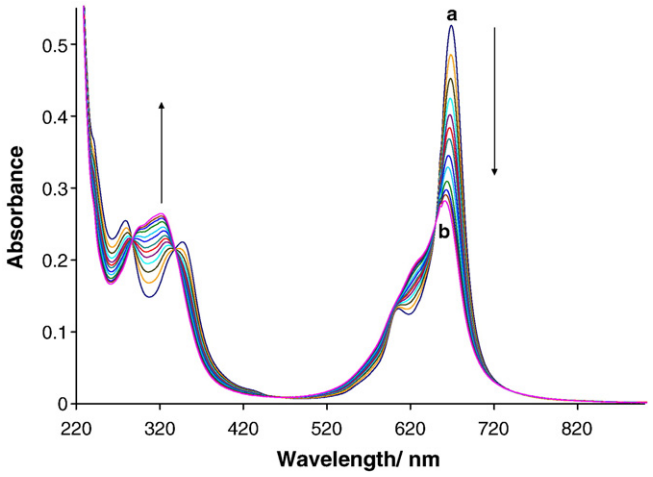


Fig. 1. Repetitive scan for the interaction of captopril with Co(III)TSPcNO in water: (a) spectrum immediately after mixing (λ_{max} = 669 nm); (b) 25 min after mixing (λ_{max} = 661 nm). [CapSH] = 50 μM, [Co(III)TSPcNO] = 5 μM, θ = 20.6 °C and [H_2EDTA^{2-}] = 50 μM.

RSNOs also absorb in the wavelength range, 330–340 nm [18], but the amount formed in this case (10^{-6} M) cannot be easily quantified spectrally due to the very small micromolar concentration levels of NO being transferred from Co(III)TSPcNO. The main spectral region of interest is completely masked by the spectrum of the strongly absorbing complex.

Reports have indicated that some thiolate anions can reduce the Co(II)TSPc to form Co(I)TSPc with the formation of a thiolate-complex intermediate [19]. No spectral changes were observed during the investigation of the interaction of the thiols in question with Co(II)TSPc, strongly supporting the idea that these thiols have no effect on the Co(II)TSPc complex within the concentration ranges studied. This observation ruled out any interaction between the excess thiol in solution and the Co(II)TSPc that was formed.

There were no further changes seen in the absorbance spectra of the interaction of Co(III)TSPcNO and thiols on the completion of the formation of monomer/dimer system of Co(II)TSPc. Hence, we conclude that this reaction is irreversible. The concentration of NO released into the system is too small to cause any further reaction with the complex.

3.2. Stoichiometry

Spectrophotometric titrations were carried out at 669 nm to determine the stoichiometry of the reaction. The stoichiometric data for the reaction between Co(III)TSPcNO and thiols at 25.1 °C show that the conversion of 1 mol of Co(III)TSPcNO requires 1 mol of thiol, in all cases, to complete the reaction. Fig. 2 shows the stoichiometric plot for the reaction between captopril and Co(III)TSPcNO. The figures depicting the stoichiometric plots for GSH, NACL and LCEE are shown as Figs. S4–S6 in the supplementary data.

Since the reaction is strongly suspected to be irreversible under the current experimental conditions, we expect that the S-nitrosothiol formed will eventually decompose releasing NO^+ in the system which is not reactive towards Co(II)TSPc.

3.3. Kinetics and mechanisms for the reaction between CoTSPcNO and thiols

The reaction between Co(III)TSPcNO and thiols was carried out under aerobic conditions and according to the following parameters: $0.929 \le 10^4$ [CapSH] ≤ 5.26 mol dm^{-3}, $20.6 \le \theta \le 37.3$ °C; $0.540 \le 10^4$ [GSH] ≤ 5.40 mol dm^{-3}, $20.5 \le \theta \le 37.4$ °C; $1.01 \le 10^4$ [LCEE] ≤ 5.04 mol dm^{-3}, $25.2 \le \theta \le 37.9$ °C; $2.54 \le 10^4$ [NALC] ≤ 21.8 mol dm^{-3}, $20.6 \le \theta \le 37.0$ °C, [H_2EDTA^{2-}] = 50 μM and [Co(III)TSPcNO] = 5 μM.

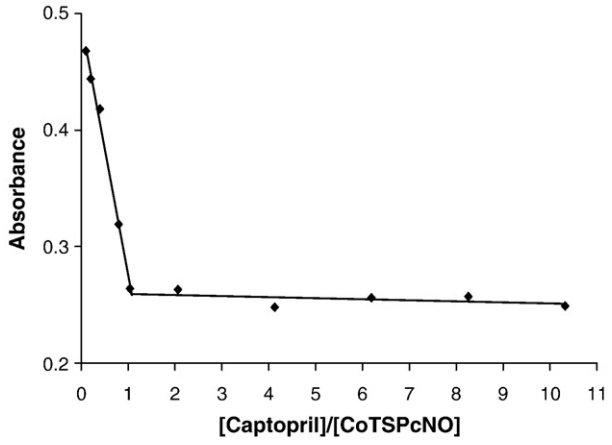


Fig. 2. Stoichiometric plot for the reaction of captopril with Co(III)TSPcNO. [Co(III)TSPcNO] = 5 μM, [H_2EDTA^{2-}] = 50 μM, θ = 25.1 °C, λ = 669 nm.

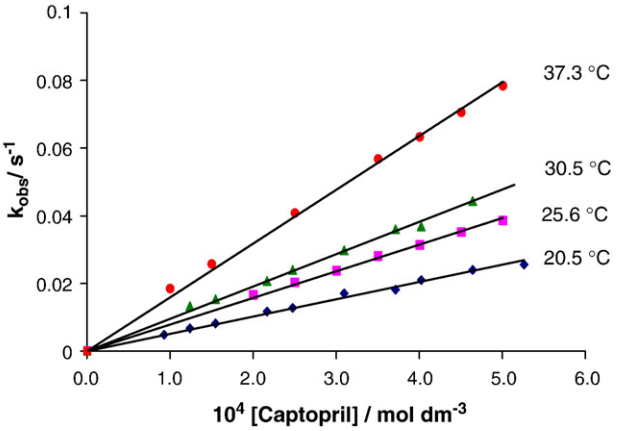


Fig. 3. Plot of observed rate constants (k_{obs}) vs. [CapSH] for the interaction with Co(III)TSPcNO in water at various temperatures. [Co(III)TSPcNO] = 5 μM, [H_2EDTA^{2-}] = 50 μM, $3.43 \le pH \le 4.16$.

Pseudo-first order rate constants are the average of at least two kinetic runs with associated errors of less than 5%.

The rates of the decomposition of [(NO)Co(III)TSPc]$^{4-}$ were determined at 669 nm and monitored on the Hewlett Packard 8453 Diode Array spectrophotometer (see the Experimental section). Under pseudo-first order conditions, the Co(III)TSPcNO/RSH interactions showed a uniphasic reaction.

3.4. Co(III)TSPcNO and RSH interaction

Pseudo-first order rate constants were obtained from the absorbance/time traces, which were analysed as single-exponential function using the Statgraphics 4.0 software in the nonlinear regression analysis mode. The rate of the reaction of Co(III)TSPcNO with the RSH form of the thiol is shown in Eq. (5), and the expression for the corresponding pseudo-first order rate constants is given in Eq. (6).

$$\text{Rate} = -\frac{d[\text{Co(III)TSPcNO}]}{dt} = k[\text{Co(III)TSPcNO}][\text{RSH}] \quad (5)$$

$$k_{obs} = k[\text{RSH}] \quad (6)$$

Plots of k_{obs} vs. [RSH] were linear for all the thiols studied, showing good first order dependency. The captopril data at various temperatures, are displayed in Fig. 3. The corresponding figures for the other thiols are in the supplementary material. The second order rate constants were determined from the slopes of these plots. Unfortunately, it was not possible to study the effect of pH variation on the rate constants, due to the interaction of buffers with the complex. However, all transnitrosation reactions studied previously have been found to be driven by the protonated species of the thiols [20,21]. The unbuffered solutions contained higher concentrations of the thiols in the RSH form, since the pH of the solution was below the pK_a values of the SH group [21].

Spectral analysis by Griess' reaction [17] confirmed the possible formation of the corresponding RSNO. On analysis of the Co(III)TSPcNO/CapSH solution, it was found that there is significantly less NO_2^- than what is expected from complete decomposition of the Co(III)TSPcNO. It is clear from the repetitive scan (Fig. 1), that the Co(III)TSPcNO loses NO completely, and it was confirmed that the NO is transferred to captopril, forming S-nitrosocaptopril which is very stable [22] in aqueous media. S-nitrosocaptopril was detected and confirmed in the reaction medium with LC/MS experiments. The ion at $m/z = 245$, corresponds to M–H$^+$, where M = CapSNO. The chromatogram and mass spectrum are displayed in Figs. S10 and S11, respectively, in the supplementary data.

Table 1
Second order rate constants and activation parameters for the interaction of Co(III) TSPcNO with thiols at physiological temperature.

Thiol	$k/M^{-1}\ s^{-1}$	$\Delta H^{\ddagger}/$ kJ mol^{-1}	$\Delta S^{\ddagger}/J\ K^{-1}\ mol^{-1}$
CapSH	159 ± 2.3	47.6 ± 4.9	-50 ± 10
GSH	66.7 ± 1.3	41.6 ± 2.7	-75.8 ± 9.0
LCEE	258 ± 8	37.7 ± 2.3	-90.6 ± 8.0
NALC	40.1 ± 0.6	31.2 ± 2.1	-113 ± 7.0

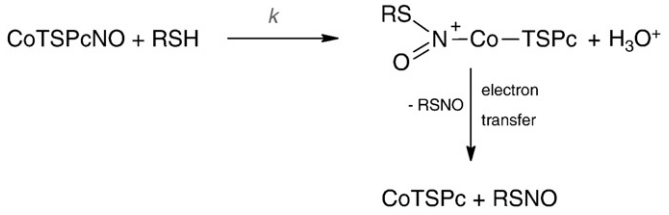
Greiss' analysis of the product solutions of all the thiol systems confirmed that the amount of NO_2^- present is inversely related to the order of stability of the respective S-nitrosothiol formed—SNOCap> GSNO>SNAC>SNCEE [23–26]. A decreasing concentration of NO_2^- was found in the product RSNO solution as follows; SNCEE>SNAC>GSNO> SNOCap, which gives further support to the transnitrosation mechanism.

3.5. Activation parameters and structure–activity relationship

The second order rate constants (LCEE>CapSH>GSH>NALC) for the various thiols, were obtained at different temperatures, by linear regressional analysis from the slopes of the plots of k_{obs} against [RSH]. Eyring's equation was used to evaluate the activation parameters ($\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$) listed in Table 1. The enthalpies of activation are not very large ($\Delta H^{\ddagger} = 47.6 \pm 4.9$, 41.6 ± 2.7, 37.7 ± 2.3, 31.2 ± 2.1 kJ mol^{-1}), reflecting the relative ease with which the reaction proceeds, and decrease along the series: CapSH>GSH>LCEE>NALC. The entropy of activation ($\Delta S^{\ddagger}$) is negative for all the thiols, and decreases along the series: CapSH>GSH>LCEE>NALC, mirroring the trend in $\Delta H^{\ddagger}$. The negative values of $\Delta S^{\ddagger}$, strongly support an associative type mechanism, in which the metal complex and the thiol come together to form a very organized and compact transition state, which facilitates the efficient transfer of the NO moiety. Activation parameters for the transnitrosation reactions between the nitroprusside anion and *L*-cysteine [27] ($\Delta H^{\ddagger} = 49 \pm 3$ kJ mol^{-1}, $\Delta S^{\ddagger} = -116 \pm 11$ J K^{-1} mol^{-1}), and between S-nitroso-*N*-acetyl-*D*,*L*-penicillamine (SNAP) and captopril [28] ($\Delta H^{\ddagger} = 49 \pm 2$ kJ mol^{-1}, $\Delta S^{\ddagger} = -32 \pm 2$ J K^{-1} mol^{-1}) compare favourably to those from this work, and support the transnitrosation mechanism.

The most probable mechanism is proposed in Scheme 1, where formation of the transition state is facilitated by polar interactions between the (NO) and (SH) moieties of the interacting species. The slightly acidic environment in the reaction medium ensures that the thiolate anion (RS^-) will not be free for a direct nucleophilic attack on the N-atom in the NO moiety in Co(III)TSPcNO.

The stability of the various transition states involving the different thiols, should be affected by the inductive effect from the different substituents on different thiols, and the steric bulk in the vicinity of the –SH moiety. Molecular-dynamic simulations would be useful here, in revealing the finer details of the intimate mechanism, and will be looked at in the future. However, the non-reactivity of *N*-acetyl-*D*,*L*-penicillamine, a tertiary thiol, is strong support that steric bulk in the region of the –SH moiety, hinders, and can prevent, the transnitrosation reaction between CoTSPc(NO) and thiols.



Scheme 1. Proposed mechanism for the interaction of RSH with Co(III)TSPcNO.

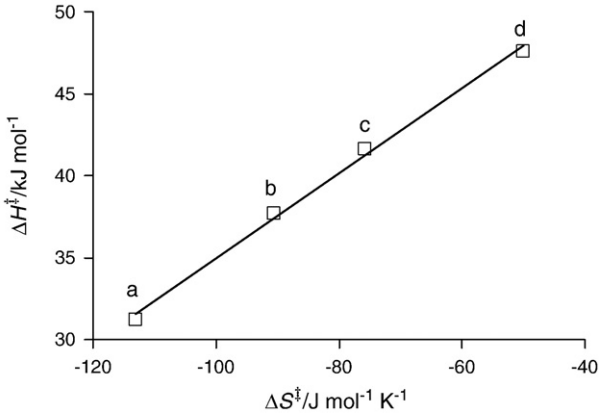


Fig. 4. Isokinetic plot ($\Delta H^{\ddagger}$ vs. $\Delta S^{\ddagger}$) for the transnitrosation of NALC (a), LCEE (b), GSH (c) and CapSH (d) by Co(III)TSPcNO.

The experimental activation parameters were employed to generate an isokinetic plot ($\Delta H^{\ddagger}$ vs. $\Delta S^{\ddagger}$), shown in Fig. 4. for the reduction of $[Co(III)TSPc(NO)]^{4-}$ by the thiols in question.

$$\Delta H^{\ddagger} = \Delta G_0^{\ddagger} + \beta_0 \Delta S^{\ddagger} \tag{7}$$

Eq. (7) was utilized for this analysis, where $\Delta G_0^{\ddagger}$ is the intrinsic free energy of activation, and β_o is the isokinetic temperature. This plot gave an excellent fit ($R^2 = 0.997$) which strongly supports the idea that all the thiols react via the same mechanism, irrespective of the type of thiol being used. The average (common) free energy of activation, $\Delta G^{\ddagger} = 61 \pm 1$ kJ mol^{-1} and the isokinetic temperature, $\beta_o = 260 \pm 11$ K were obtained from the intercept and slope, respectively.

4. Conclusions

The mechanism of interaction of Co(III)TSPc(NO) with free thiols has been found to be similar to other transnitrosation reactions reported in the literature. The mechanism involves transfer of NO to thiol, accompanied by intramolecular electron transfer. The reaction product, S-nitrosocaptopril, has been identified in the kinetic solution for the first time by using HPLC attached to mass spectrometric detector.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bpc.2009.01.014.

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