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Ru^{III}(EDTA) mediated S-nitrosylation of cysteine by nitrite†

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Reported here is the first example of a ruthenium(III) complex $[Ru^{III}(EDTA)(H_2O)]^-$ ($EDTA^{4-}$ = ethylenediaminetetraacetate) that mediates S-nitrosylation of cysteine in the presence of nitrite at pH 4.5 (acetate buffer) and results in the formation of $[Ru^{III}(EDTA)(SNOCy)]^-$. The kinetics of the reaction was studied by stopped-flow and rapid-scan spectrophotometry as a function of [Cysteine], $[NO_2^-]$ and pH (3.5–8.5). Formation of $[Ru^{III}(EDTA)(SNOCy)]^-$, the product of the S-nitrosylation reaction, was identified by ESI-MS experiments. A working mechanism in agreement with the spectroscopic and kinetic data is presented.

Introduction

Many physiological activities of NO within every major organ system are accomplished by selective modification of protein cysteine residues to form S-nitrosocysteine. In this post-translational modification, S-nitrosylation plays a crucial role in the regulation of the protein function by acting as signal effectors.[1] Furthermore, essential roles for S-nitrosylation have also been implicated in almost all major functions related to cardiac signaling,[2] and signal transduction, thereby promoting synaptic damage, cell death and neurodegeneration.[3]

Studying the reaction of $[Ru^{III}(EDTA)(H_2O)]^-$ ($EDTA^{4-}$ = ethylenediaminetetraacetate) with these two important biomolecules, *viz.* cysteine and NO, has been our enduring interest. We reported earlier that $[Ru^{III}(EDTA)(H_2O)]^-$ reacts rapidly with cysteine (CySH) to form the red coloured $[Ru^{III}(EDTA)(CyS)]^{2-}$ complex characterized by a band at 510 nm (λ_{max} = 3330 M^{-1} cm^{-1}).[4a] We also explored the ability of $[Ru^{III}(EDTA)(H_2O)]^-$ towards inhibition of cysteine protease,[4a] and protein tyrosine phosphatase activity in a mechanism that involves the binding of the CyS residue of the catalytic domain of the enzymes.[4b] Our contribution to the chemistry of the Ru(EDTA)/NO system, signifying the role of the Ru-EDTA type of complex as a NO scavenger, is also well documented in the literature.[5] Very recently, we have shown that Ru(EDTA) could mediate O-atom transfer from nitrite (NO_2^-) to cysteine (CySH) leading to the formation of the $[Ru^{III}(EDTA)(NO)]$ complex and cysteine sulfenic acid (CySOH). However, cysteine sulfenic acid undergoes a further rapid reaction with another molecule of cysteine that results in the formation of cysteine as the ultimate oxidation product at higher pH (>5.0).[6] In the present work we demonstrate S-nitrosylation of the coordinated cysteine in $[Ru^{III}(EDTA)(CyS)]^{2-}$ by nitrite. As far as we know, this is the first report on the use of any ruthenium complex for mediating S-nitrosylation of cysteine.

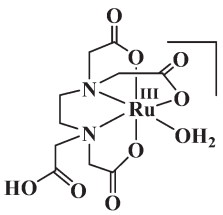

Fig. 1 Pictorial representation of the $[Ru^{III}(HEDTA)(H_2O)]$ complex.

Experimental

Materials

$K[Ru^{III}(HEDTA)Cl]\cdot 2H_2O$ was synthesized according to the published procedure.[7] Anal. calculated for $K[Ru^{III}(HEDTA)Cl]\cdot 2H_2O$: C 24.0, H 3.42, N 5.59; Found. C 23.8, H 3.45, N 5.63. IR, ν/cm^{-1}: 1720 (free –COOH), 1650 (coordinated $-COO^-$). UV-Vis in H_2O: λ_{max}/nm (ε_{max}/M^{-1} cm^{-1}): 283 (2800 ± 50), 350 sh (680 ± 10). The $K[Ru^{III}(HEDTA)Cl]$ complex rapidly converts into the $[Ru^{III}(HEDTA)(H_2O)]$ complex (Fig. 1) when dissolved in water.[8,9] The sixth coordination site of the ruthenium complex is occupied either at low pH by a water molecule

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or at high pH by a hydroxide ion. The pK_a values related to the acid-dissociation equilibria of the pendant carboxylic acid arm and the coordinated water molecule are 2.4 and 7.6, respectively, at 25 °C.[8,9] All other chemicals used were of A.R. grade. Doubly distilled H_2O was used throughout the experiments.

Instrumentation

UV-vis spectral measurements were carried out on a Varian Model Cary 100 spectrophotometer using 1 cm path standard quartz cells. Stopped-flow experiments were performed on a Hi-Tech SF-61 SX2 (TgK Scientific Ltd) spectrophotometer using a 1 cm optical path length. The reaction of $[Ru^{III}(EDTA)(CyS)]^{2-}$ with nitrite (NO_2^-) was followed at 510 nm. Single wavelength kinetic profiles were collected in the photomultiplier mode by following the disappearance of $[Ru^{III}(EDTA)(CyS)]^{2-}$ at 510 nm, and data were processed using the Hi-Tech KinetAsyst 3 software. Time-resolved UV-vis spectra were recorded on a rapid scan diode array spectral attachment (KinetaScan). The solution temperature was maintained within ±0.1 °C using a circulating water bath (JEIO TECH RW-1025G). All the instruments were thermostated at the desired temperature (±0.1 °C). The pH of the solutions was measured with a Mettler Delta 350 pH meter. Acetate, phosphate and borate buffers were used to adjust the pH of the experimental solutions. Kinetic data are presented as an average of several kinetic runs (at least 5–8) and were reproducible within ±4%. ESI-MS measurements were performed on a UHR-TOF Bruker Daltonik (Bremen, Germany) maXis, capable of a resolution of at least 40 000 FWHM in the group of Prof. Ivana Ivanović-Burmazović, University of Erlangen-Nürnberg. Detection was in the negative-ion mode and the source voltage was 4.5 kV. The flow rate was 300 μL h^{-1}. The drying gas (N_2), to aid solvent removal, was kept at 180 °C. The instrument was calibrated prior to every experiment *via* direct infusion of the Agilent ESI-TOF low concentration tuning mixture, which provided an m/z range of singly charged peaks up to 2700 Da in both ion modes. The aim of the ESI-MS experiments was to identify the intermediates and products of the reaction.

Results and discussion

Addition of $NaNO_2$ to the red solution of $[Ru^{III}(EDTA)(CyS)]^{2-}$ (prepared by mixing the solutions of $[Ru^{III}(EDTA)(H_2O)]^-$ and cysteine in an equimolar ratio) at pH 4.5 (10 mM acetate buffer) resulted in a rapid drop of the spectral features (Fig. 2) characteristic of the $[Ru^{III}(EDTA)(CyS)]^{2-}$ complex. The observed spectral changes (Fig. 2) are attributed to the S-nitrosylation of the coordinated cysteine in $[Ru^{III}(EDTA)(CyS)]^{2-}$ by NO_2^- under the specified conditions.

The formation of $[Ru^{III}(EDTA)(SNOCy)]^{2-}$ in the reaction of $[Ru^{III}(EDTA)(CyS)]^{2-}$ and NO_2^- was confirmed by ESI-MS studies. The recorded spectrum for the solution containing the $[Ru(EDTA)(H_2O]^-$ complex alone showed a characteristic signal for $[Ru(EDTA)]^-$ at m/z = 389.96 (Fig. 3a). Addition of cysteine to the solution of $[Ru^{III}(EDTA)(H_2O)]^-$ produced a signal at m/z = 510.98 (Fig. 3b), which can be assigned to the formation of the red coloured $[Ru^{III}(EDTA)(CyS)]^{2-}$. On addition of NO_2^- to this red solution, the peak (at m/z = 510.98) corresponding to $[Ru^{III}(EDTA)(CyS)]^{2-}$ disappeared and formation of $[Ru^{III}(EDTA)(SNOCy)]^{2-}$ (m/z = 540.98) could be clearly evidenced (Fig. 3c). In all cases the experimental and simulated spectra were in excellent agreement.

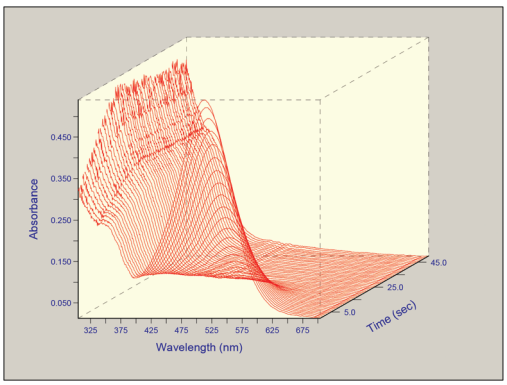


Fig. 2 UV-Vis spectral changes for the reaction of $[Ru^{III}(EDTA)(CyS)]^{2-}$ (0.2 mM) with NO_2^- (2.0 mM) at 25 °C, pH 4.5 (10 mM acetate buffer).

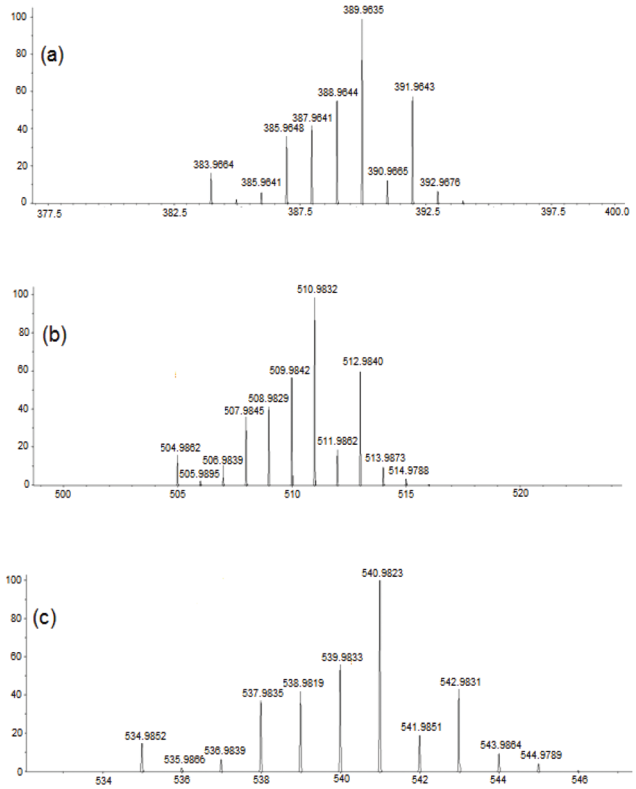


Fig. 3 Summary of ESI-MS data: spectrum recorded (a) for solution of 1 × 10^{-4} M $[Ru^{III}(edta)H_2O]^-$ (b) after addition of 0.2 mM cysteine to the solution of (a), and (c) after addition of 2 mM of $NaNO_2$ to the solution of (b) in 10 mM acetate buffer at pH = 4.5.

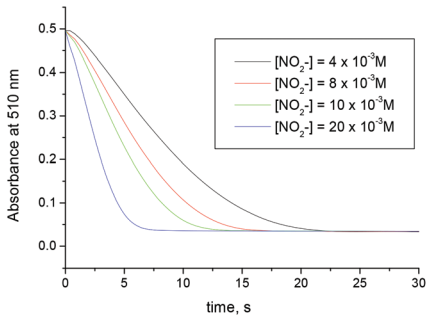


Fig. 4 Effect of nitrite concentration on the reaction of $[Ru^{III}(EDTA)(CyS)]^{2-}$ (0.2 mM) with NO_2^- at 25 °C, pH 4.5 (10 mM acetate buffer).

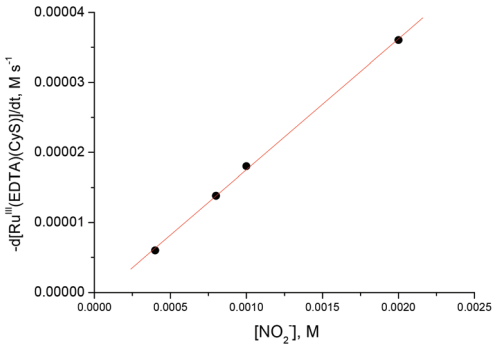


Fig. 5 Plot of the rate of the reaction of $[Ru^{III}(edta)(CyS)]^{2-}$ with NO_2^- *versus* $[NO_2^-]$ at 25 °C, $[Ru^{III}(EDTA)(CyS)]^{2-}$ = 0.2 mM, pH 4.5 (10 mM acetate buffer).

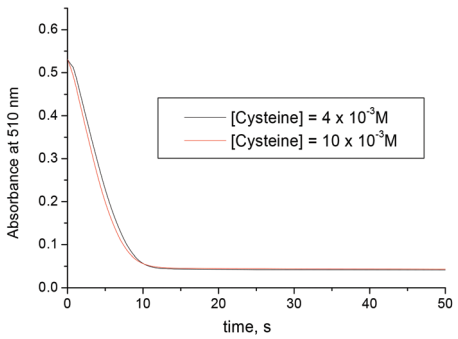


Fig. 6 Effect of cysteine concentration on the reaction of $[Ru^{III}(EDTA)(CyS)]^{2-}$ with NO_2^- at 25 °C, pH 4.5 (10 mM acetate buffer). $[Ru^{III}]$ = 0.2 mM, $[NO_2^-]$ = 10 mM.

On the basis of the above observations, we performed a series of stopped-flow kinetic studies in which the nitrite concentration and pH were systematically varied at constant temperature. The effect of the nitrite concentration on the rate of the reaction was studied at pH 4.5, and representative kinetic traces recorded at 510 nm are shown in Fig. 4.

The small induction period observed at lower nitrite concentration may be explicable in terms of the formation of [Ru(EDTA)(NO)] in the reaction of $[Ru(EDTA)(H_2O)]^-$ and NO_2^-.[6] The $[Ru^{III}(EDTA)(Cys)]^{2-}$ is pre-formed by reacting $[Ru^{III}(EDTA)(H_2O)]^-$ with cysteine (CysH) in an equimolar ratio, and under the specified conditions there still exists a small amount of free $[Ru^{III}(EDTA)(H_2O)]^-$ ($K = 3 \times 10^5$ M^{-1}).[4a] Since, NO_2^- to NO^+ conversion involves acid–base chemistry and at lower pH it exists as $(NO^+{\cdot}H_2O)$, the reacting species (NO^+/NO_2^-) present in the reacting system under the employed conditions react with the free $[Ru^{III}(EDTA)(H_2O)]^-$ (in equilibrium with $[Ru^{III}(EDTA)(Cys)]^{2-}$) leading to the formation of the $[Ru^{III}(EDTA)(NO)]$ species[6] before effecting S-nitrosylation of coordinated cysteine in $[Ru^{III}(EDTA)(Cys)]^{2-}$ species though present in bulk. Formation of $[Ru^{III}(EDTA)(NO)]$ species (showing a peak at *m*/*z* 419.95 in the spectrum) along with the formation of $[Ru^{III}(EDTA)(Cys)]^{2-}$ is evidenced by ESI-MS studies (see Fig. S1 in ESI†). However, at higher nitrite concentration the rate of formation of $[Ru^{III}(EDTA)(NO)]$ is much faster,[6] and no induction period is therefore visible in Fig. 4.

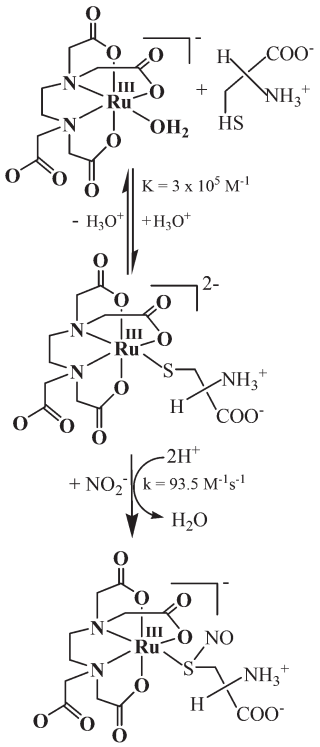
Under the employed conditions (as specified in Fig. 4), the rate of the reaction estimated from the maximum slope of the kinetic traces in Fig. 4, increases linearly with increasing NO_2^- concentration (see Fig. 5). We also studied the effect of the cysteine concentration (in excess over the $[Ru^{III}(EDTA)(H_2O]^-$ complex) on the rate of the reaction at a fixed $[NO_2^-]$. All the absorbance–time traces shown in Fig. 6 exhibit no initial induction period, signifying that the reformation of $[Ru^{III}(EDTA)(H_2O)]^-$ *via* hydrolysis of $[Ru^{III}(EDTA)(SNOCy)]^-$ does not occur rapidly, and as a consequence formation of $[Ru^{III}(EDTA)(CyS)]^{2-}$ (through the reaction of $[Ru^{III}(EDTA)(H_2O)]^-$ with cysteine) is not continued for further recycling at higher cysteine concentration. Moreover, the rate of the disappearance of $[Ru^{III}(EDTA)(CyS)]^{2-}$ estimated from the slope of the kinetic traces (Fig. 6) is seemingly independent of [CyS]. This suggests that the rate of the S-nitrosylation of coordinated cysteine in $[Ru^{III}(EDTA)(CyS)]^{2-}$ is independent of the cysteine concentration under the specified conditions.

Very recently we have reported the proton assisted interconversion of $[Ru^{III}(EDTA)(NO_2)]^{2-}$ to $[Ru^{III}(EDTA)(NO^+)]^0$ at pH 4.5 in a mechanism that involves oxide ion (O^{2-}) transfer from coordinated nitrite to H^+.[6] Based on the above observations, a working mechanism is proposed in Scheme 1 for the $[Ru^{III}(EDTA)(H_2O)]^-$ mediated S-nitrosylation of cysteine.

In the proposed reaction scheme, binding of cysteine to the Ru(III) center occurs through an aqua substitution step, producing the red coloured $[Ru^{III}(EDTA)(Cys)]^{2-}$. The red colour is characteristic for sulfur to ruthenium charge transfer[10] in the $[Ru^{III}(EDTA)(CyS)]^{2-}$ complex. In the subsequent step, the nitrite ion effects S-nitrosylation of coordinated cysteine plausibly by generating NO^+ species (in a proton assisted pathway) which directly attacks the sulfur atom of the coordinated cysteine in $[Ru^{III}(EDTA)(CyS)]^{2-}$ to form the S-nitrosylated $[Ru^{III}(edta)(SNOCy)]^-$ product complex (Scheme 1).

At a constant pH (4.5) the following rate-law can be derived for the reactions outlined in Scheme 1 on the basis that the rate-determining disappearance of the $[Ru^{III}(EDTA)(CyS)]^{2-}$ complex is monitored at 510 nm.

$$\text{Rate} = kK[\text{CyS}][NO_2^-][Ru^{III}(\text{edta})(H_2O)^-]/(1 + K[\text{CyS}]) \quad (1)$$



Scheme 1 Mechanism for the $[Ru^{III}(EDTA)(H_2O)]^-$ mediated S-nitrosylation of cysteine by the nitrite ion.

Considering the high value of K (3×10^5 M^{-1}),[4a] eqn (1) can be simplified to (2) under the specified conditions since $1 \ll K[CyS]$.

$$\text{Rate} = k[NO_2^-][Ru^{III}(edta)(H_2O)^-] \quad (2)$$

The rate law in (2) can account for the zero order behaviour with respect to cysteine concentration (Fig. 6). The linear dependence of the rate on $[NO_2^-]$ (Fig. 5) in the presence of excess $[NO_2^-]$ ($[NO_2^-] \gg [Ru^{III}]$), is in line with the suggested mechanism. The value of the second-order rate constant (k) estimated from the slope of the plot of rate *versus* $[NO_2^-]$ (as shown in Fig. 5) is 93 ± 4 M^{-1} s^{-1} at 25 °C and pH 4.5.

It is apparent from Scheme 1 that S-nitrosylation of coordinated cysteine is governed by the pH of the reaction mixture. Considering that the pK_a value of proton dissociation of HNO_2 is 3.4 at 25 °C,[11] pH dependence studies were carried out in the range 4.5 to 8.5, so that NO_2^- would be the major reacting species in the selected pH range. The observed decrease in the reaction rate with increasing pH (Fig. 7), agrees with the mechanistic proposal involving binding of NO^+ (which is the key species) generated from the proton coupled oxide ion (O^{2-}) transfer from nitrite to H^+. However, at higher pH (> 6) a slow disappearance of the band at 510 nm (Fig. 7) was still observed. This is presumably associated with aerial oxidation of coordinated cysteine at higher pH. Control experiments performed in the absence of nitrite revealed similar observations with regard to the disappearance of the spectral

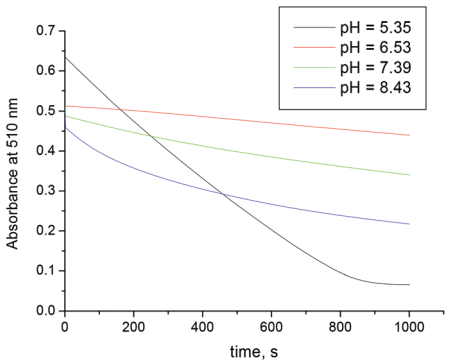


Fig. 7 Effect of pH on the reaction of $[Ru^{III}(EDTA)(CyS)]^{2-}$ (0.2 mM) with NO_2^- (10 mM) at 25 °C.

features of the $[Ru^{III}(EDTA)(CyS)]^{2-}$ complex and further support the above claim.

Conclusions

In summary, we report the first example of a ruthenium(III) complex capable of mediating S-nitrosylation of cysteine in the presence of sodium nitrite at pH 4.5. Direct S-nitrosylation of cysteine by nitrite involving participation of NO^+ under strong acidic conditions was reported earlier.[12,13] A third-order rate constant of 13.0 M^{-2} s^{-1} was reported for the formation of the S-nitrosocysteine (CySNO) in aqueous solution of cysteine (CySH) and sodium nitrite at pH lower than 3.5.[13] Comparison of the rate data reveals that the rate of the $[Ru^{III}(EDTA)(H_2O)]^-$ mediated S-nitrosylation of cysteine is faster than the direct S-nitrosylation of cysteine by nitrite. The above findings suggest that $[Ru^{III}(EDTA)(H_2O)]^-$ not only binds NO,[14] but is also able to store NO as a S-nitroso (SNO) conjugate of the $[Ru^{III}(EDTA)(CyS)]^{2-}$ complex mimicking heme-assisted S-nitrosylation of a proximal thiolate in a nitric oxide transport protein.[15]

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