Kinetics of nitric oxide scavenging by ruthenium(iii) polyaminocarboxylates: novel therapeutic agents for septic shock

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The reaction of two representative ruthenium(III) polyaminocarboxylates with nitric oxide are rapid ($k > 10^7$ M^{-1} s^{-1} at pH 7.4 and 7.3 °C) forming tight ($K = 10^8$ M^{-1}) ruthenium(II) mononitrosyls.

In the last decade nitric oxide (NO) has been found to be an essential component in many physiological processes and dysfunction in NO metabolism has been associated with a number of disease states such as epilepsy, arthritis, hypertension and septic shock.[1,2] In particular, overproduction of NO is implicated in the catastrophic drop in blood pressure associated with septic shock. This hypotension does not always respond well to conventional therapy such as intravenously administered adrenaline and dopamine.[3] Novel therapeutic approaches have largely concentrated on the development of inhibitors of the enzyme, inducible nitric oxide synthase (iNOS).[4] An alternative, however, is to remove NO by use of a specific, high affinity, non-toxic scavenger.

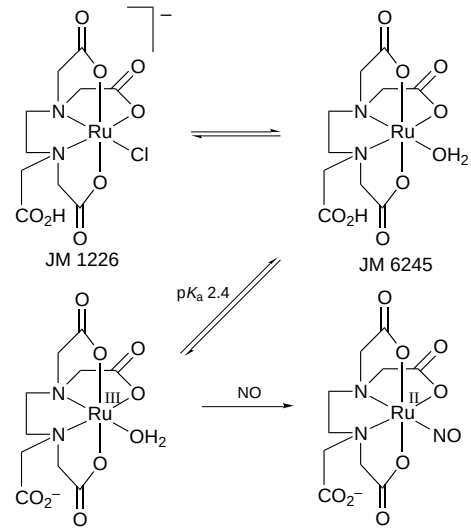
NO is a well characterised ligand for transition metals, having a high affinity for ferrous haem, indeed the biological effects of NO in regulation of blood pressure are brought about by the interaction of NO with the haem iron of the enzyme guanylate cyclase.[1] Ruthenium has a high affinity for NO and forms more nitrosyls than any other metal.[5] A strategy has been adopted in which ruthenium is chelated with suitable ligands which confer water solubility, allowing rapid *in vivo* clearance and unlike iron, low toxicity, whilst providing an available binding site for NO. The polyaminocarboxylates satisfy these requirements as ligands.

We have shown that the ruthenium(iii) polyaminocarboxylates K[Ru(Hedta)Cl],[6] JM1226, and [Ru(Hedta)(H_2O)],[6,7] JM6245 (Scheme 1) will reduce the level of nitrite in the cell culture medium of RAW264 macrophage cells stimulated to produce NO, and reverse lipopolysaccharide (LPS) induced hypotension in rats.[8,9]

Here, we report the NO binding properties of JM1226 and JM6245, JM1226 is water soluble at mm concentrations, forming a pale yellow solution in phosphate buffer over the pH range 6.5–8.0. Storage of JM1226 in phosphate buffer at pH 7.4 for more than 5 h at room temperature resulted in a change in the absorption spectrum due to formation of a dark green species. This green species is attributed to formation of a mixed valence $Ru^{III}Ru^{IV}$ μ-oxo dimer.[10] All experiments were therefore performed with freshly prepared solutions.

The absorption spectra of JM1226 at all pH values examined (6.5–8.0) are identical to the corresponding spectra for JM6245, indicating that the chloride is rapidly replaced in aqueous solution and that the mechanism of reaction of JM1226 with NO proceeds *via* the formation of the aqua species, JM6245, Scheme 1.

On addition of an aqueous NO solution to a solution of JM1226 a small change is observed in the near-UV region of the spectrum, leading to a loss of absorbance at 290 nm and an increase in absorbance below 270 nm, with an isosbestic point at 272 nm. We have utilized this absorbance change to determine the binding stoichiometry of NO to Ru^{III} and to obtain an estimate of the binding affinity. Fig. 1 shows a titration of JM1226 (100 μm) with NO. The inset to Fig. 1 demonstrates that NO forms a 1 : 1 adduct with JM1226/



Scheme 1 The proposed mechanism for the reaction of JM1226 with NO involves formation of the aqua complex, JM6245. Substitution at the aqua position is thought to proceed by associative ligand substitution[7] to give the ruthenium(ii) mononitrosyl.

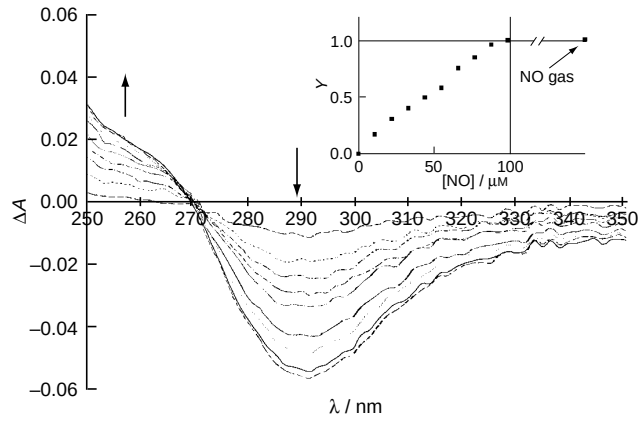


Fig. 1 Difference spectrum of JM1226 in phosphate buffer (50 mm) at pH 7.4 titrated against an aqueous NO solution of known concentration calculated by titration of ferrous myoglobin with the NO solution.[11] Inset: the binding curve of JM1226 (100 μm) and NO is calculated for the absorbance change at 290 nm and indicates a 1 : 1 Ru–NO complex is formed. The fractional saturation, Y, is defined as $Y = (A - A_0)/(A_\infty - A_0)$, where A_0 and A_∞ are absorbances in the absence and presence of saturating NO and A is the absorbance after the addition of a sub-saturating concentration of NO.

JM6245. The affinity is high and cannot be determined from this figure directly, however the linearity of the binding curve approaching saturation indicates that the binding constant is $> 10^8$ m^{-1}. These findings are consistent with formation of a strong Ru–NO bond.[5]

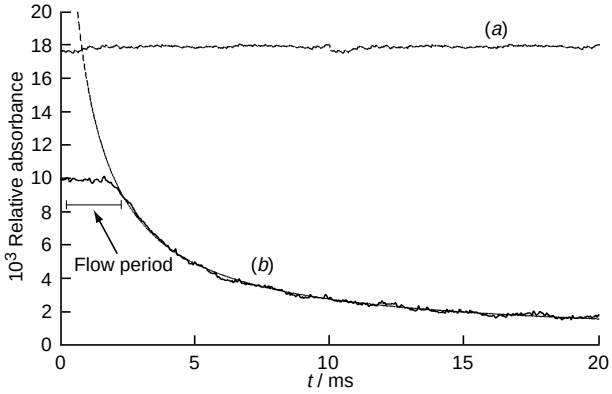


Fig. 2 Change in absorbance of an anaerobic solution of JM1226 (25 μm) with time, in the absence (*a*) and presence (*b*) of NO (25 μm) in phosphate buffer (50 mm), pH 7.4 at 7.3 °C. The fit is to a second-order rate equation, traces are an average of six measurements. Spectra were recorded on an Applied Photophysics DX17 stopped-flow spectrometer with a measured deadtime of 1 ms.

Table 1 Calculated second-order rate constants from stopped-flow experiments. All experiments were conducted in phosphate buffer (50 mm) at the pH and temperature stated. Errors represent standard deviation, $n = 6$, in each case

Compound	pH	*T*/°C	10^{-7} *k*/m^{-1} s^{-1}
JM1226	8	7.7	3.33 ± 0.17
JM1226	7.4	7.3	2.24 ± 0.05
JM1226	6.5	7.4	1.39 ± 0.01
JM6245	8	7.2	3.29 ± 0.14
JM6245	7.4	7.2	1.95 ± 0.06
JM6245	6.5	7.5	2.18 ± 1.06

The binding of NO by JM1226/6245 is extremely rapid, Fig. 2, Table 1. As expected JM1226 and 6245 yield, within experimental error, the same rate constants and the reactions display little, if any, pH dependence. At room temperature most of the reaction occurs within the dead time of the apparatus (1 ms), hence the rate measurements were made at 7 °C. At this temperature we see a rapid NO concentration-dependent loss in absorbance, the amplitude of which is consistent with the spectral change on formation of the Ru–NO complex, Fig. 1. At wavelengths below the isobestic point (272 nm, Fig. 1) we observed an increase in absorbance (not shown) with the same course as that shown in Fig. 2 confirming that the process depicted in the figure is a result of the rapid binding of NO to the ruthenium complex. At pH 7.4 and 7.3 °C the rate constant is *ca*. 2×10^7 m^{-1} s^{-1}, we therefore estimate that at physiological temperature the rate constant will exceed 10^8 m^{-1} s^{-1}.

We have shown that at physiological pH JM1226/JM6245 binds NO tightly and rapidly to form a stable 1 : 1 ruthenium(ii) mononitrosyl. These properties confirm the pharmacological observations in a model of LPS induced endotoxic shock,[8] that ruthenium(iii) polyaminocarboxylate complexes have potential as NO scavengers in NO mediated diseases.

References

1 S. Moncada, R. M. J. Palmer and E. A. Higgs, *Pharmacol. Rev*., 1991, **43**, 109.
2 C. Nathan, *FASEB J*., 1993, **6**, 3051.
3 M. P. Glauser, G. Zanetti, J.-D. Baumgartner and J. Cohen, *Lancet*, 1991, **338**, 732.
4 J. F. Kerwin, Jr., J. R. Lancaster, Jr. and P. L. Feldman, *J. Med. Chem*., 1995, **38**, 4343.
5 F. Bottomley, *Coord. Chem. Rev*., 1978, **26**, 7.
6 A. A. Diamantis and J. V. Dubrawski, *Inorg. Chem*., 1981, **20**, 1142.
7 T. Matsubara and C. Creutz, *Inorg. Chem*., 1979, **18**, 1956.
8 S. P. Fricker, E. Slade, N. A. Powell, B. A. Murrer, I. L. Megson, G. D. Kennovin, S. T. Bisland, M. Loveland and F. W. Flitney, *The Biology of Nitric Oxide*, Portland Press, London, 1996, Part 5, p. 330.
9 S. P. Fricker, *Platinum Met. Rev*., 1995, **39**, 150.
10 J. Zhou, W. Xi and J. K. Hurst, *Inorg. Chem*., 1990, **29**, 160.
11 J. Torres and M. T. Wilson, *Methods Enzymol*., 1996, in the press.

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