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Interaction of $[Ru^{III}(edta)(H_2O)]^-$ with amino acids in aqueous solution. Equilibrium, kinetic and protease inhibition studies †

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The interaction of $[Ru^{III}(edta)(H_2O)]^-$ (edta = ethylenediaminetetraacetate) with amino acids, *viz*. glycine, L-cysteine and *S*-methylcysteine, was investigated potentiometrically and kinetically. The concentration distribution of various complex species was evaluated as a function of pH. Kinetic data obtained as a function of [amino acid], temperature (5.0 to 45.0 °C) and pressure at a fixed pH of 6.0, reveal that the formation of $[Ru^{III}(edta)(Am)]^-$ (Am = amino acid) occurs *via* a rapid amino acid concentration dependent complex-formation reaction of $[Ru^{III}(edta)(H_2O)]^-$, followed by a slow amino acid concentration independent ring-closure step. The kinetic data and activation parameters are interpreted in terms of an associative interchange mechanism and discussed in reference to data reported for closely related systems in the literature. Enzyme inhibition studies revealed that $[Ru^{III}(edta)(H_2O)]^-$ effectively inhibits the cysteine protease activity in papain and bromalein enzymes.

Introduction

Cysteine proteases, familiar as thiol protease in older literature,[1] have recently been discovered in viruses of poliomyelitis, hepatitis A,[2,3] and their pathological role[4–7] in brain trauma, muscular dystrophy, arthritis, cardiac ischaemia, and Alzheimer's disease due to the degradation of the concerned proteins by the enzyme, is explored. Furthermore, it has been suggested that the cysteine proteases have a control influence on HIV-1, myocardial repair, and the peridontal disease, cytomeglavirus (herpes).[8] Unlike the mechanism of serine proteases, the molecular basis of protein degradation by cysteine proteases is not fully understood,[8] though it is certain that the imidazole group of histidine polarizes the thiol (SH) group of cysteine to produce the thiolate ion, which attacks the carbonyl group of the peptide bond and results in the cleavage of the protein. Due to a similar proteolytic mechanism,[9] most of the inhibitors[10–15] which are organic compounds, mainly aldehyde and ketone derivatives, react with both serine and cysteine proteases. In order to achieve selective inhibition for cysteine proteases, Cysteine Protease Inhibitor (CPI) should have an active site which could be selectively highly reactive with the cysteine residue of the enzyme to produce an inert covalent enzyme–inhibitor complex. Such type of inhibitors may be suitable as drugs for treatment of the diseases described above. However, the use of metal complexes in this regard is conspicuously absent in the literature.

An increasing awareness has developed in recent years of the potential of Ru(edta) type complexes in biological processes.[16–18] A recent report[19] on the potential of the $[Ru^{III}(edta)(H_2O)]^-$ complex to serve as a model system for the physiological NO synthase reaction further illustrates this fact. The ethylenediaminetetraacetate (edta) ligand is somewhat similar in its donor character to many metalloenzymes, which utilize carboxylate, amine or imidazole donors from Asp, Glue, Lys or His amino acids to bind to the metal centre. An important chemical aspect is that $[Ru^{III}(edta)(H_2O)]^-$ exhibits a high reactivity towards displacement of coordinated water.[20,21] This occurs in a direct manner and offers the advantage of facile formation of mixed-ligand complexes. Our current research interest[22–26] is partly focused on kinetic and mechanistic aspects of the interaction of $[Ru(edta)(H_2O)]^-$ with biologically relevant molecules, including NO, to explore the possibility of using $[Ru(edta)(H_2O)]^-$ in a variety of bioinorganic applications. In this respect, the present study reports for the first time the cysteine protease inhibition property of $[Ru(edta)(H_2O)]^-$ in papain and bromalein enzymes (both contain a cysteine SH-group). In order to rationalize the inhibition by $[Ru(edta)(H_2O)]^-$, we also report the results of potentiometric and kinetic studies of the interaction of $[Ru^{III}(edta)(H_2O)]^-$ with glycine, cysteine and *S*-methylcysteine. Although the kinetics of the reaction of $[Ru(edta)(H_2O]^-$ with cysteine and other thio-amino acids leading to the formation of S-coordinated species has been reported before,[22,27] the system was revisited since in the course of this work we clearly observed two reaction steps, something that was not observed in the earlier studies.

Experimental

$K[Ru^{III}(Hedta)Cl]\cdot 2H_2O$ was prepared by a published procedure[28] and characterized by elemental analysis and spectral data which were in agreement with data reported in the literature.[28] The complex rapidly aquates to the aqua complex when dissolved in water, and thus exists predominantly in its most labile form $[Ru^{III}(edta)(H_2O)]^-$ in the pH range 5 to 6.[20,21] All other chemicals used were of AR grade and doubly distilled H_2O was used to prepare all solutions.

The pH titrations were performed on an automatic titrator (Metrohm 702 SM Titrino). The acid dissociation constants of $[Ru^{III}(edtaH)(H_2O)]$ (involving uncoordinated COOH and coordinated H_2O), glycine, cysteine and *S*-methylcysteine were determined by titrating 0.05 mM (30 mL) samples of each with a standard NaOH solution (0.091 M). Formation constants of the mixed-ligand complexes were obtained by titrating a solution containing $[Ru^{III}(edtaH)(H_2O)]$ and the amino acid ligands

† Electronic supplementary information (ESI) available: kinetic plots and a scheme showing the reaction between $[Ru^{III}(edta)(H_2O)]^-$ and cysteine. See http://www.rsc.org/suppdata/dt/b2/b208495n/

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(Am) in a 1 : 1 concentration ratio. Calculations of equilibrium data were performed using the program MINIQUAD-75,[29] whereas the species distribution diagrams were obtained using the program SPECIES.[30]

The pH of the solutions was measured with a Mettler Delta 350 pH meter. Phosphate buffer was used to maintain the pH at 6.0 during the kinetic studies at ambient pressure, whereas Bis-Tris buffer was used to maintain this pH at elevated pressure since its dissociation constant is not affected by pressure.[31] Absorption spectra were recorded on Cary 1G or Shimadzu spectrophotometers equipped with thermostatted cell compartments. Kinetic measurements were carried out on an Applied Photophysics SX 18.MV stopped-flow instrument coupled to an on-line data acquisition system. At least eight kinetic runs were recorded under all experimental conditions. The substitution reaction was followed at wavelengths where appreciable spectral changes between the reactant and product species exists, *viz*., 390 nm for glycine, 510 nm for cysteine and 335 nm for *S*-methylcysteine. Kinetic measurements at high pressure were carried out using a homemade, high pressure stopped-flow system.[32] Kinetic traces were analysed with the OLIS KINFIT program (Bogart, GA). All the instruments were thermostatted at the desired temperature (±0.1 °C). The values of the observed rate constants (k_{obs}) are presented as the average value of the kinetic runs with an average reproducibility of ±4%.

Enzyme inhibition studies were performed with papain (Sigma) and bromalein (pine apple extract) enzymes using azocasein as substrate and $[Ru^{III}(edta)(H_2O)]^-$ as inhibitor. Stock solutions of enzyme (blank) and enzyme–inhibitor were prepared as follows. A stock solution of the papain enzymes was prepared by dissolving 3 mg papain in 500 μL water, whereas the bromalein enzyme stock solution contained 100 μL bromalein in 400 μL water. The enzyme–inhibitor solutions were prepared by dissolving 5 mg of $K[Ru^{III}(Hedta)Cl]$ (0.01 mmol) in another set of blank solutions (500 μL) of enzyme. Experimental solutions were pH 7.5 (phosphate buffer) and preincubation at 25.0 °C for 30 min prior to the studies. Then they were subjected to the assay according to the protocol given later (seeTable 4). The solutions in each set were then incubated for one hour at 37.0 °C. After incubation, 5% trichloroacetic acid (500 μL) was added to precipitate the unreacted protein and kept undisturbed for 30 min. They were then subjected to centrifugation, followed by the addition of 0.5 N NaOH (500 μL) to the supernatant liquid. Finally, the absorbance of the resulting solutions was recorded at 440 nm.

Results and discussion

Equilibrium studies

The acid dissociation constants of $[Ru^{III}(Hedta)(H_2O)]$ and the complex-formation constants with glycine, cysteine and *S*-methylcysteine in aqueous solution, determined under the selected experimental conditions, are summarized in Table 1. The reported pK_a values are in good agreement with data reported in the literature.[33] The difference in the pK_1 (2.96) and pK_2 (7.76) values, corresponding to the proton dissociation of uncoordinated $-CO_2H$ and coordinated H_2O, as compared to those reported before (pK_1 = 2.37 and pK_2 = 7.63),[34] can be ascribed to the difference in ionic strength.

Analysis of the titration data for the Ru^{III}(edta)–amino acid system shows the formation of 1 : 1 complexes. In the case of cysteine, it coordinates through the thiol group with the amine group being protonated (species 111). Upon increase in pH, deprotonation occurs to give the 110 species. The pK_a of the protonated species (111) is given by eqn. (1),

$$pK_a = \log \beta_{111} - \log \beta_{110} \quad (1)$$

Table 1 Equilibrium constants for the formation of glycine, cysteine and *S*-methylcysteine complexes with $[Ru^{III}(edta)(H_2O)]^-$ at 25.0 °C and I = 0.1 M (sodium triflate)

System	l	p	q^a	$\log \beta^b$
Ru(edta)	1	0	1	2.96 (0.01)
	1	0	−1	7.76 (0.01)
Glycine	0	1	1	9.65 (0.01)
	1	1	0	6.35 (0.06)
Cysteine	0	1	1	10.34 (0.01)
	0	1	2	18.52 (0.01)
	1	1	0	11.05 (0.06)
	1	1	1	17.63 (0.03)
S-Methylcysteine	0	1	1	8.81 (0.01)
	1	1	0	6.53 (0.06)

[a] l, p and q are the stoichiometric coefficients corresponding to Ru^{III}(edta) complex, amino acid ligand and H^+, respectively. [b] Standard deviations are given in parentheses.

and the estimated value was found to be 6.58. For *S*-methylcysteine and glycine the values obtained are 6.53 and 6.35, respectively. The stability constants of the 1 : 1 complexes decrease in the order cysteine > *S*-methylcysteine > glycine. The higher stability in the case of cysteine is ascribed to the stronger bonding of the thiolate group. Coordination through the sulfur atom in the case of *S*-methylcysteine is evidenced by the higher stability constant than that of glycine, even though the amine group in *S*-methylcysteine is less basic than in glycine. These results, in particular the observation of an acid dissociation equilibrium for the amine group in the $[Ru^{III}(edta)(gly)]^-$ complex, support the htpothesis that glycine coordinates through its carboxylate group to the Ru(III) center. The speciation diagram for the various complex species is shown in Fig. 1. The monoprotonated cysteine complex (111) predominates with a degree of formation of 81% at pH 5.5 (Fig. 1a), and the corresponding deprotonated form (110) starts to form at pH 5.0 and increases in concentration with increasing pH. The concentration distribution diagram of *S*-methylcysteine (Fig. 1b) and glycine (Fig. 1c) exhibit a similar trend. The high stability constants observed in all cases, suggest that the amino acid ligands under investigation behave as bidentate ligands and form mixed chelate products through the opening of one of the coordinated carboxylate arms of the edta ligand.

Kinetic studies. *Interaction of glycine with $[Ru^{III}(edta)(H_2O)]^-$.* Preliminary experiments in which the UV-Vis spectrum was scanned in the range 200 to 800 nm showed that at pH 6.0 glycine reacts rapidly with $[Ru^{III}(edta)(H_2O)]^-$, which is the main species in solution at this pH based on the above cited pK_a values. In addition, earlier studies[20–26] have clearly demonstrated this species to be by far the most reactive Ru^{III} complex in solution. The change in absorbance at 390 nm was employed for the kinetic studies. Kinetic traces recorded by mixing solutions of 1×10^{-3} M $[Ru^{III}(edta)(H_2O)]^-$ and glycine (0.05–0.2 M) in the stopped-flow instrument at pH 6.0 (phosphate or Bis-Tris buffer) and I = 0.1 M ($NaNO_3$) were found to be double exponential in nature (see Fig. SI-1, ESI). The observed first order rate constant (k_{obs}) for the first reaction step was obtained by analysing the kinetic trace on a shorter time scale (10 s) separately for a single exponential as shown in Fig. SI-2 (ESI). The rate constant increases linearly with increasing glycine concentration and shows a negligible intercept as shown in Fig. SI-3 (ESI), indicating that neither a reverse nor parallel reaction contributes significantly. This kinetic behaviour can be expressed in terms of the rate law given in eqn. (2).

$$k_{obs} = k_1[gly] \quad (2)$$

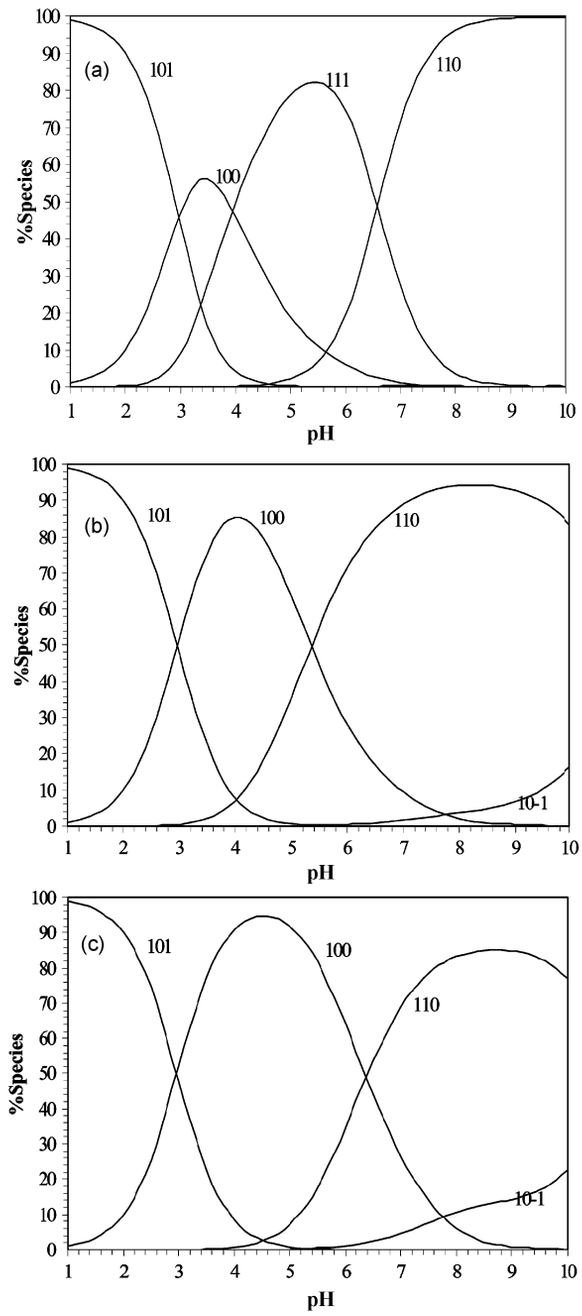


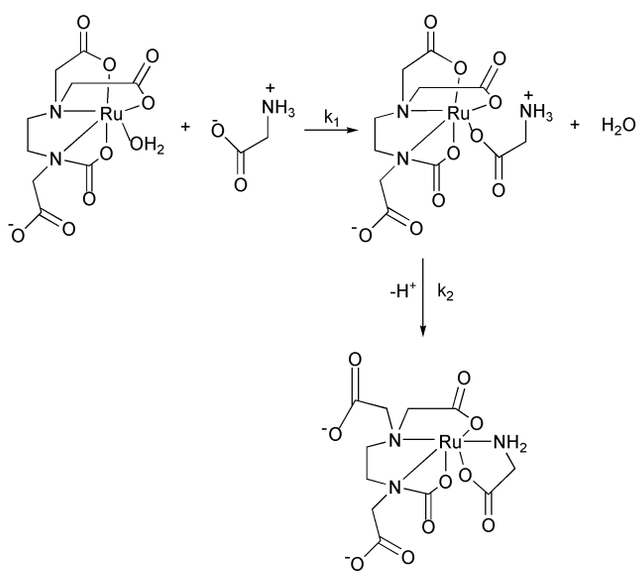
Fig. 1 Species distribution for various species as a function of pH in the (a) Ru(edta)–cysteine system; (b) Ru(edta)–*S*-methylcysteine system; and (c) Ru(edta)–glycine system (at a concentration of 1.66 mM for Ru(edta)), $I = 0.1$ M (sodium triflate).

The first order rate constants for the subsequent slower reaction step, determined over longer reaction times, were found to be independent of the glycine concentration (Table 2). Considering the analogous kinetic behaviour reported for the reaction of $[Ru^{III}(edta)(H_2O)]^-$ with bidentate ligands, *viz*. 2-marcaptopyridine[35] and nucleotides,[25] leading to the formation of mixed chelate product complexes, the present kinetic results can be interpreted in terms of Scheme 1. Rapid formation of the mono-coordinated complex through substitution of coordinated water by the carboxylate group of glycine, followed by a ring closure step (independent of the glycine concentration) in which the protonated amine group of glycine undergoes deprotonation (the acidity of the protonated amine group of glycine is increased due to its coordination to the Ru(III) center) and coordinates to the metal center by displacing one carboxylate arm of the edta ligand (Scheme 1).

Table 2 Observed rate data for the reaction of $[Ru^{III}(edta)(H_2O)]^-$ with amino acids (Am) at pH 6.0 and $I = 0.1$ M ($NaNO_3$)

Am	[Am]/M	T/°C	k_{obs}^1/s^{-1}	k_{obs}^2 [a]/s^{-1}
Glycine [b]	0.025	25.0	0.43 ± 0.01	0.009 ± 0.001
	0.050		0.88 ± 0.02	0.009 ± 0.001
	0.075		1.29 ± 0.03	0.012 ± 0.002
	0.10		1.74 ± 0.05	0.013 ± 0.002
Cysteine [c]	0.005	25.0	1.32 ± 0.02	0.051 ± 0.008
	0.010		2.47 ± 0.01	0.055 ± 0.005
	0.015		3.95 ± 0.07	0.049 ± 0.004
	0.020		5.34 ± 0.15	0.055 ± 0.007
S-Methylcysteine [c]	0.005	25.0	1.51 ± 0.02	0.40 ± 0.05
	0.010		2.49 ± 0.01	0.43 ± 0.02
	0.015		3.38 ± 0.09	0.44 ± 0.05
	0.020		4.54 ± 0.15	0.48 ± 0.03

[a] The values of k_{obs}^2 decrease or increase slightly with increasing [Am]. However, the data are considered to be independent of [Am], since the change in absorbance associated with the second step of the reaction is very small and the error limits are substantially larger than observed for the first reaction step. [b] $[Ru^{III}] = 5 \times 10^{-4}$ M, [c] $[Ru^{III}] = 2.5 \times 10^{-4}$ M.



Scheme 1

The reaction between $[Ru^{III}(edta)(H_2O)]^-$ and glycine was studied as function of temperature and pressure, and the corresponding kinetic data and activation parameters are summarized in Table 3. The good agreement between the values of $\Delta H_1^{\neq} = 57 \pm 1$ kJ mol^{-1}, $\Delta S_1^{\neq} = -31 \pm 5$ J K^{-1} mol^{-1} and $\Delta V_1^{\neq} = -3.3 \pm 0.2$ cm^3 mol^{-1} observed in the present study and those reported for the substitution of $[Ru^{III}(edta)(H_2O)]^-$ with other ligands,[20,21] clearly supports the operation of an associative interchange mechanism in the reaction of $[Ru^{III}(edta)(H_2O)]^-$ with glycine.

Interaction of cysteine and S-methylcysteine with $[Ru^{III}(edta)(H_2O)]^-$. The interaction of cysteine and other thio-amino acids with $[Ru^{III}(edta)(H_2O)]^-$, resulting in the formation of S-coordinated $[Ru^{III}(edta)(SR)]$ (RSH = thioamino acids), was studied before.[22,27] The complexes containing different thiolates ($[Ru^{III}(edta)(SR)]$) are reportedly stable ($K = ca.\ 10^5$ M^{-1}).[27] As $[Ru^{III}(edta)(H_2O)]^-$ is a very weak oxidant ($E_{1/2}$ corresponding to the Ru^{III}/Ru^{II} redox couple is -0.26 V *vs*. SCE),[20] the bound thiolates were found to be redox unreactive under the specified conditions.[27] Although a detailed mechanistic investigation on the interaction of RSH with $[Ru^{III}(edta)(H_2O)]^-$, performed over a wide pH range (1.2–10.0), had been reported earlier,[22,27] the results obtained for glycine in the preceding section, prompted us to reinvestigate the reaction of $[Ru^{III}(edta)(H_2O)]^-$

Table 3 Rate and activation parameters for the reaction of $[Ru^{III}(edta)(H_2O)]^-$ with different amino acids

Am	T/°C	P/MPa	k_1/M^{-1} s^{-1}	k_{-1}/s^{-1}
Glycine[a]	10.0	0.1	4.8 ± 0.1	
	15.0		7.1 ± 0.2	
	20.0		11.8 ± 0.1	
	25.0		17.4 ± 0.5	
	30.0		24.0 ± 0.9	
	35.0		37.0 ± 0.8	
	20.0	10	10.5 ± 0.3	
		50	11.1 ± 0.2	
		90	11.8 ± 0.3	
		130	12.3 ± 0.1	
	$\Delta H_1^{\neq} = 57 \pm 1$ kJ mol^{-1}, $\Delta S_1^{\neq} = -31 \pm 5$ J K^{-1} mol^{-1}, $\Delta V_1^{\neq} = -3.3 \pm 0.2$ cm^3 mol^{-1}			
Cysteine[b]	5.0	0.1	49.9 ± 1.3	
	10.0		79.3 ± 1.3	
	20.0		168 ± 1	
	25.0		263 ± 5	
	30.0		326 ± 6	
	15.0	10	147 ± 7	
		50	162 ± 5	
		90	176 ± 2	
		130	186 ± 3	
	$\Delta H_1^{\neq} = 51 \pm 2$ kJ mol^{-1}, $\Delta S_1^{\neq} = -28 \pm 7$ J K^{-1} mol^{-1}, $\Delta V_1^{\neq} = -4.7 \pm 0.4$ cm^3 mol^{-1}			
S-Methylcysteine[b]	5.0		18 ± 2	0.15 ± 0.03
	15.0		62 ± 4	0.36 ± 0.06
	25.0		200 ± 8	0.49 ± 0.10
	35.0		430 ± 6	1.46 ± 0.08
	40.0		630 ± 30	2.1 ± 0.4
	45.0		790 ± 40	3.6 ± 0.5
	$\Delta H_1^{\neq}$/kJ mol^{-1}		68 ± 4	54 ± 5
	$\Delta S_1^{\neq}$/J K^{-1} mol^{-1}		25 ± 12	−66 ± 16

[a] $[Ru(edta)H_2O] = 5 \times 10^{-4}$ M, pH 6.0, and $I = 0.1$ M ($NaNO_3$). [b] $[Ru(edta)H_2O] = 2.5 \times 10^{-4}$ M, pH 6.0 and $I = 0.1$ M ($NaNO_3$).

with cysteine. As observed before, mixing solutions of $[Ru^{III}(edta)(H_2O)]^-$ and cysteine at pH 6.0 rapidly produced an intense colour ($\lambda_{max} = 510$ nm) associated with the formation of S-coordinated $[Ru^{III}(edta)(SR)]$ (RSH = cysteine). However, the kinetic trace for the reaction of 2.5×10^{-4} M $[Ru^{III}(edta)(H_2O)]^-$ with cysteine (0.005–0.02 M) at pH 6.0 and $I = 0.1$ M clearly displayed two exponential functions as shown in Fig. 2a. The kinetic trace for the first step, corresponding to the formation of the S-coordinated species, was analysed separately on a shorter time scale (2 s) for two exponentials (shown in Fig. 2b), since there is also interference between the two reaction steps on this time scale. The observed rate constant (k_{obs}) for the first step increases linearly with increasing cysteine concentration with a negligible intercept (shown in Fig. SI-4, ESI), indicating that neither a reverse nor a parallel reaction contributes significantly to the observed kinetics. The value of k_1 was found to be 271 ± 10 M^{-1} s^{-1} at 25 °C, as compared to 332 at pH = 5.0 and 170 M^{-1} s^{-1} at pH 5.5 found before.[22,27] The negligible intercept found in the present study agrees with that reported earlier[22] and the small value (0.57×10^{-3} s^{-1}) estimated for the reverse reaction from equilibrium data.[27] Table 2 summarizes the kinetic data obtained for the first step. The thermal activation parameters obtained in the present study (see Table 3) are in reasonable agreement with those reported in the previous studies.[22,27] In addition, the reaction is characterized by a significant negative volume of activation ($\Delta V_1^{\neq} = -4.7 \pm 0.4$ cm^3 mol^{-1}) estimated from the pressure dependence studies (see Table 3), which is in good agreement with the values ranging from −4.1 to −12.2 cm^3 mol^{-1} reported for the substitution of $Ru^{III}(edta)$ type complexes with various thio ligands.[36,37] These values support the operation of an associative interchange mode of activation.

Analysis of the slower second step (Fig. 2a) yielded rate constants that are independent of the cysteine concentration (see Table 2). The overall kinetic behaviour of the reaction of $[Ru^{III}(edta)(H_2O)]^-$ with cysteine at pH 6.0, in which rapid formation of the S-coordinated species takes place in a cysteine concentration dependent pathway, followed by the cysteine concentration independent ring-closure step (Scheme I, ESI) in which the protonated amine group of cysteine deprotonates and coordinates to the Ru^{III} center by displacing one carboxylate arm of the edta ligand. Under these experimental conditions, there is a further slow decay in absorbance at 510 nm over many hours, which is ascribed to subsequent hydrolysis of the [Ru(edta)(cysteine)] complex.

The kinetics of the reaction between *S*-methylcysteine (MeSR) and $[Ru^{III}(edta)(H_2O)]^-$ was followed in the UV region at 335 nm since the solution obtained after mixing did not show any band in the visible region. The absence of a strong ligand to metal charge transfer (LMCT) band in the visible region indicates that *S*-methylcysteine cannot form a thiolate (S^-) group which is a stronger donor and shifts the LMCT band into the visible region as for cysteine. Similar to that observed for cysteine, the reaction of *S*-methylcysteine with $[Ru^{III}(edta)(H_2O)]^-$ was found to display a two exponential kinetic trace consisting of an absorbance increase followed by a slow absorbance decrease as shown in Fig. SI-5 (ESI). Kinetic results for the first step were analyzed separately as for the reactions with glycine and cysteine. Fig. 3 demonstrates that the observed rate constant increases linearly with increasing *S*-methylcysteine concentration at different temperatures (5.0 to 45.0 °C) with significant intercepts within the experimental error limits. This observation can be described by the rate law given in eqn. (3),

$$k_{obs} = k_1[MeSR] + k_{-1} \quad (3)$$

where k_1 and k_{-1} represent rate constants for the forward and reverse reactions, respectively.

The rate and activation parameters are summarized in Table 3. $\Delta H_1^{\neq}$ and $\Delta S_1^{\neq}$ were found to be 68 ± 4 kJ mol^{-1} and +25 ±

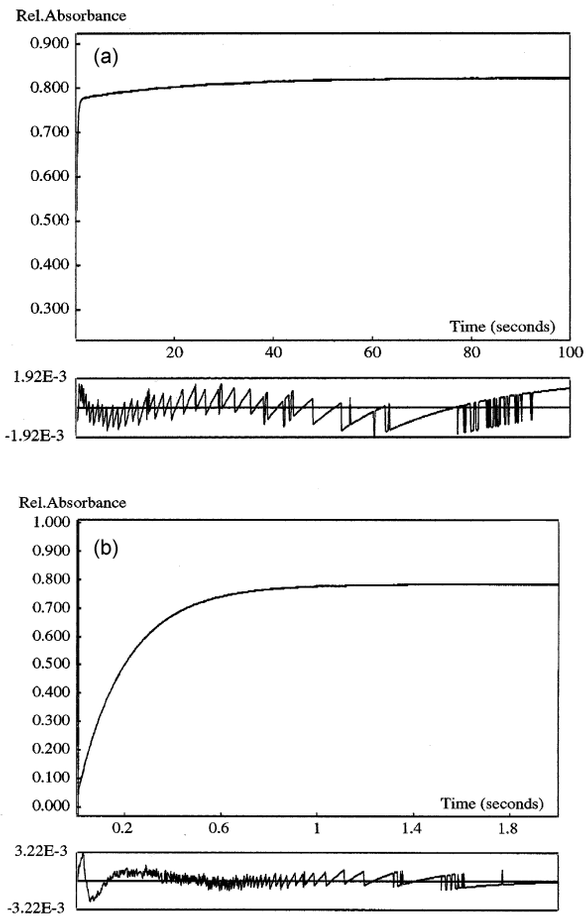


Fig. 2 (a) Typical kinetic trace for the reaction between 2.5×10^{-4} M $[Ru(edta)H_2O]^-$ and 0.02 M cysteine, pH 6.0 (phosphate buffer) and 25.0 °C. The trace was fitted to two exponentials by following the increase in absorbance at 510 nm. The lower trace represents the difference between the experimental and calculated curves. (b) Typical kinetic trace for the reaction between 2.5×10^{-4} M $[Ru(edta)H_2O]^-$ and 0.02 M cysteine, pH 6.0 (phosphate buffer) and 25.0 °C. The trace was fitted to two exponentials by following the increase in absorbance at 510 nm. The lower trace represents the difference between the experimental and calculated curves.

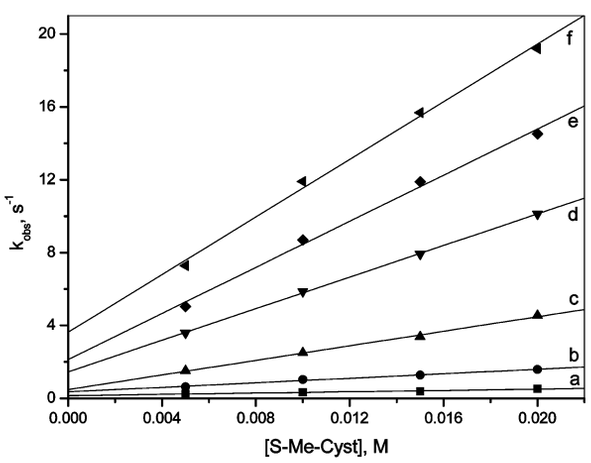


Fig. 3 Plot of k_{obs} *versus* [S–Me–Cyst] for the reaction between $[Ru(edta)H_2O]^-$ and *S*-methylcysteine as a function of temperature. Experimental conditions: $[Ru(edta)H_2O^-] = 2.5 \times 10^{-4}$ M, pH 6.0, temperature = 5.0 (a); 15.0 (b); 25.0 (c); 35.0 (d); 40 (e) and 45.0 °C (f), and $I = 0.1$ M ($NaNO_3$).

12 J K^{-1} mol^{-1}, respectively. It is known that $\Delta S^{\neq}$ is usually subjected to large error limits because of the intrinsic extrapolation involved in its determination, such that small absolute values are not significant in terms of the assignment of a reaction mechanism.[38,39] $\Delta H_{-1}^{\neq}$ and $\Delta S_{-1}^{\neq}$ for the reverse reaction were found to be 54 ± 5 kJ mol^{-1} and −66 ± 16 J K^{-1} mol^{-1}, respectively. These values support an associative character for the aquation reaction of the [Ru(edta)(Me-cysteine)] complex.

Analysis of the slower second step (Fig. SI-5, ESI) yielded rate constants that are independent of the Me-cysteine concentration (see Table 2). The overall kinetic behaviour of the reaction of $[Ru^{III}(edta)(H_2O)]^-$ with *S*-Me-cysteine at pH 6.0 is similar to that shown in the case of cysteine, in which rapid formation of the S-coordinated species takes place in a *S*-Me-cysteine concentration dependent pathway, followed by the *S*-Me-cysteine concentration independent ring-closure step in which the protonated amino group of *S*-Me-cysteine deprotonates and coordinates to the Ru(III) center by displacing one carboxylate arm of the edta ligand. Under these experimental conditions, there is a further slow decay in absorbance at 335 nm, which is presumably due to hydrolysis of the [Ru(edta)-(*S*-Me-cysteine)] complex.

Cysteine protease inhibition studies

The high affinity of the $[Ru^{III}(edta)(H_2O)]^-$ complex towards the –SH group enables a study of the possible role of Ru^{III}-(edta) type complexes in cysteine protease inhibition. In order to examine the inhibition activity of $[Ru^{III}(edta)(H_2O)]^-$, studies with papain and bromalein enzymes were performed since both of them contain a thiol group of the cysteine residue. Azocasein was used as substrate for this purpose. Enzyme inhibition studies were performed according to the protocol defined in Table 4. The results of the inhibition studies shown in Figs. 4a and 4b, clearly demonstrate that $[Ru^{III}(edta)(H_2O)]^-$

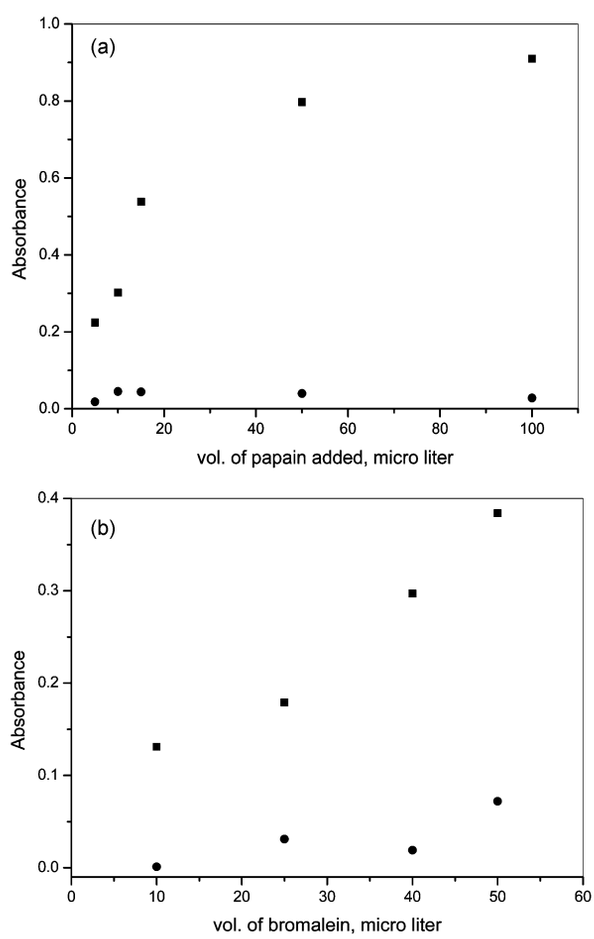
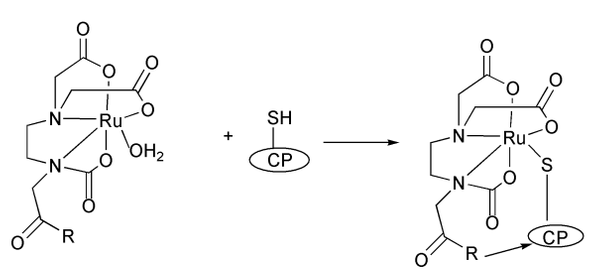


Fig. 4 Inhibition of cysteine protease activity of papain (a) and bromalein (b) enzymes by $[Ru(edta)H_2O^-]$. Squares represent non-inhibited reaction, circles represent inhibited reaction. For experimental details see text and Table 4.

Table 4 Results of cysteine protease inhibition with $[Ru^{III}(edta)(H_2O)]^-$

Enzyme	Set	Buffer added/μL	Substrate added/μL	Enzyme added/μL	Absorbance at 440 nm
Papain (without inhibitor)	Blank without enzyme	750	250	—	0.072
	Blank with enzyme	900	—	100	0.002
	1	745	250	5	0.224
	2	740	250	10	0.302
	3	735	250	15	0.538
	4	700	250	50	0.797
	5	650	250	100	0.910
Papain (with inhibitor)	Blank without enzyme	750	250	—	0.070
	Blank with enzyme containing inhibitor	900	—	100	0.003
	1	745	250	5	0.018
	2	740	250	10	0.045
	3	735	250	15	0.044
	4	700	250	50	0.040
	5	650	250	100	0.028
Bromalein (without inhibitor)	Blank without enzyme	750	250	—	0.038
	Blank with enzyme	950	—	50	0.00
	1	740	250	10	0.131
	2	725	250	25	0.179
	3	710	250	40	0.297
	4	700	250	50	0.384
Bromalein (with inhibitor)	Blank without enzyme	750	250	—	0.034
	Blank with enzyme containing inhibitor	950	—	50	0.00
	1	740	250	10	0.001
	2	725	250	25	0.031
	3	710	250	40	0.019
	4	700	250	50	0.072

appreciably inhibits the hydrolysis of azocasein by papain or bromalein under the specified conditions. The high affinity of the $[Ru^{III}(edta)(H_2O)]^-$ towards binding of the –SH group in cysteine is probably the reason for the observed protease inhibition in the present case. In order to achieve selective inhibition for cysteine proteases, the inhibitor should have a highly reactive site to bind the –SH group of the cysteine residue of the enzyme to produce an inert covalent enzyme–inhibitor complex.[22] Probably a similar mechanism is operative in the present case, where the –SH group in the cysteine residue of the papain or bromalein enzymes is bound to the Ru^{III} center through a rapid substitution reaction. Inhibition of the protease activity is thus observed due to formation of a stable Ru(edta)–enzyme complex as shown in Scheme 2.



Scheme 2 R = Peptide unit that recognizes enyme selectivity; CP = Cysteine Protease.

Conclusions

The results of the present study provide clear mechanistic information on the reaction of the $[Ru^{III}(edta)(H_2O)]^-$ complex with glycine, cysteine and *S*-methylcysteine. Values for k_1 in the case of glycine, cysteine and *S*-methylcysteine were found to be 17.4, 263 and 200 M^{-1} s^{-1}, respectively. These values show that both cysteine and *S*-methylcysteine exhibit similar rate constants that are significantly higher than for glycine. The stability constants of the 1 : 1 complexes decrease in the order cysteine > *S*-methylcysteine > glycine. The higher stability in the case of cysteine is ascribed to the more efficient binding of the thiolate donor as evidenced by the higher rate constant.

The ability of $[Ru^{III}(edta)(H_2O)]^-$ to inhibit the cysteine protease activity in both papain and bromalein enzymes could be clearly demonstrated. The high affinity of $[Ru^{III}(edta)(H_2O)]^-$ towards the –SH group reveals the possible role of Ru^{III}(edta) type complexes in cysteine protease inhibition. $[Ru^{III}(edta)(H_2O)]^-$ probably binds the –SH group in the cysteine residue of the enzyme through a rapid water displacement reaction and so inhibits the protease activity of the enzyme by forming a stable Ru(edta)–enzyme complex.

In order to achieve selective inhibition of cysteine protease, the cysteine protease inhibitor (CPC) should comprise a peptide segment for recognition by the enzyme and an active site which can react with the cysteine residue of the enzyme to produce an inert covalent enzyme–inhibitor complex.[8] Based on the results of the present studies it appears that the $[Ru^{III}(edta)(H_2O)]^-$ complex modified by linking an appropriate peptidal unit to the dangling uncoordinated carboxylate group for enzyme recognition could serve as an inhibitor in which the –SH group in the cysteine residue of the enzyme can bind the modified Ru(III) complex through a rapid substitution reaction (Scheme 2), and thus inhibit the protease activity of the enzyme by forming a stable Ru(edta)–enzyme complex. Further work related to this matter is in progress.

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