Biometals (2009) 22:771–778
DOI 10.1007/s10534-009-9224-5

Determination of the pKa value of the hydroxyl group in the α-hydroxycarboxylates citrate, malate and lactate by 13C NMR: implications for metal coordination in biological systems

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Received: 27 February 2009 / Accepted: 4 March 2009 / Published online: 14 March 2009

Abstract Citric acid is an important metal chelator of biological relevance. Citric acid helps solubilizing metals, increasing their bioavailability for plants and microbes and it is also thought to be a constituent of both the extracellular and cytoplasmic low molecular iron pools occurring in plants and vertebrates. Metal coordination by citric acid involves coordination both by the carboxylate and hydroxyl groups, of particular interest is its α-hydroxycarboxylate function. This structural feature is highly conserved in siderophores produced by evolutionarily distant species and seems to confer specificity toward Fe(III) binding. In order to understand the mechanism of metal coordination by α-hydroxycarboxylates and correctly evaluate the respective complex stability constants, it is essential to improve the knowledge about the ionisation of the alcohol group in these compounds. We have evaluated the hydroxyl p*K*a value of citric, malic and lactic acids with the objective of understanding the influence of α-carbon substitution. Studies at high pH values, utilizing ^{13}C NMR, permitted estimation of the p*K*a values for the three acids. The p*K*a (alcohol) values (14.4 for citric acid, 14.5 for malic acid, and 15.1 for lactic acid) are considerably higher than the previously reported value for citric acid (11.6) but still lower than the value of 15.5 for methanol. A comparative analysis of the three compounds indicates that different substitutions on the α-carbon introduce changes to the inductive effect experienced by the hydroxyl group thereby modulating its ionisation behaviour. Comparison with the siderophore rhizoferrin, which p*K*a (alcohol) values were confirmed to be 10 and 11.3, suggests that intramolecular hydrogen bonding may also aid in the hydroxyl ionisation by stabilizing the resulting anion. Studies of metal coordination by α-hydroxycarboxylates should take these factors into account.

Keywords Citrate · Malate · Lactate · pKa · Siderophores · α-Hydroxycarboxylate

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Introduction

Citric acid is a ubiquitous molecule, essential for aerobic life. The activity of citric acid as a metal chelator is central to its importance in living systems, with the Krebs cycle step catalyzed by aconitase being dependent on the binding of citric acid to the iron–sulphur cluster of the enzyme (Villafranca and Mildvan 1972). Further, citric acid has been proposed to be a constituent of the low molecular weight cytosolic iron pool (Bakkeren et al. 1985). It is clear that a sound understanding of iron citrate interactions

is essential for a complete description of cellular iron metabolism.

In the blood plasma, citric acid exists at a concentration of 0.1 mM and was shown to be the major ligand of the non-transferrin-bound iron (NTBI) pool existing in haemochromatosis and thalassaemia major patients (Grootveld et al. 1989). The presence of NTBI facilitates infection (Barton et al. 2006) as citric acid is used as an exogenous siderophore by bacteria. *E. coli* possess a specific transport system for ferric citrate (Harle et al. 1995) and *Bradhyrhizobium japonicum* releases citric acid under iron-deficient growth conditions (Guerinot et al. 1990). In plants citric acid is closely linked with iron acquisition being the main iron ligand for the iron transport in the xylem sap (Pitch et al. 1991). The binding of other metals such as copper (Field et al. 1974), cobalt (Matzapetakis et al. 2000a), lead (Kourgiantakis et al. 2000), manganese (Matzapetakis et al. 2000b), aluminium (Matzapetakis et al. 1999) and gallium (Matzapetakis et al. 2001) to citrate has also been described and may prove to be of biological relevance.

From many X-ray crystallography studies it becomes apparent that citrate coordinates metals through its carboxylate and hydroxyl groups (Matzapetakis et al. 1998, 1999, 2000b, 2001; Shweky et al. 1994; Bino et al. 1998; Gautier-Luneau et al. 2005; Strouse et al. 1977). Citric acid (Fig. 1a) is a tricarboxylic acid with three readily dissociable protons: $pKa_1 = 3.13$, $pKa_2 = 4.76$ and $pKa_3 = 6.40$ (Martell and Smith 1977). However, confusion exists on matters relating to the ionisation of citric acid hydroxyl group. Most speciation studies treat citric acid as having only three ionisable groups, but some authors consider it to possess four protonation sites, the value for the hydroxyl pKa being taken as 11.6 (Migal and Sychen 1958). This confusion leads to the utilization of different speciation models and is reflected in the variation of citrate—iron stability constants.

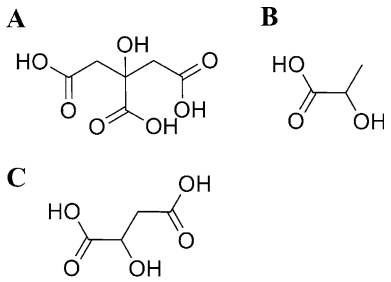

Fig. 1 Chemical structure of **a** citric acid, **b** malic acid and **c** lactic acid

Considering the importance of iron coordination by citric acid in biological systems and also for industrial applications (e.g., in soft drinks, as food preserving agent and as a cleaning and polishing agent for metal surfaces) we have reassessed the pKa value of the hydroxyl group of citric acid and the related α-hydroxycarboxylates malic acid (Fig. 1b) and lactic acid (Fig. 1c). We failed to find any pKa value between 9 and 12 using potentiometric titration in any of these acids. However, ^{13}C NMR studies at pH values between 10 and 14 permitted the estimation of the hydroxyl group protonation constant for the three compounds. The results obtained demonstrate that the alcohol proton in α-hydroxycarboxylates is not as acidic as previously described, but dissociates more easily than simple aliphatic hydroxyl protons. We suggest that the fourth pKa of citric acid is ideally suited to act as a selective mechanism for the preferable chelation of trivalent metals such as Fe(III) and Al(III) over the binding of divalent metals such as Cu(II) or Zn(II). This selectivity may explain why several species of bacteria produce siderophores containing the α-hydroxycarboxylic function as one of the iron binding moieties (Drechsel and Winklemann 2000).

Materials and methods

Materials

Citric acid monohydrate (C1909) and 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt (DSS) (178837) were obtained from Aldrich Chemicals. L-Lactic (69771) and L-Malic (02290) acids were from Fluka. Rhizoferrin was obtained from EMC microcollections GmbH, Germany. For titrations, standard potassium hydroxide aqueous solution with a nominal concentration of 10 M was used (J/6630C/90, Fisher Scientific UK). Ionic strength values were adjusted using potassium chloride (A. C. S. reagent, VWR). Throughout the study HPLC grade water was used after boiling for 15 min in order to minimize carbon dioxide content.

Potentiometric titration

In this study an automatic titration system was utilized comprising an autoburette (Metrohm Dosimat 765 1 ml syringe) and Mettler Toledo MP230 pH meter with Metrohm pH electrode (6.0133.100) and a reference electrode (6.0733.100). The temperature of the test solutions was maintained in a thermostatic jacketed titration vessel at 25 ± 0.1°C using a Techne TE-8 J temperature controller.

For titrations at pH values below 12, citric acid (68.76 mg) in 10 ml 0.1 M potassium chloride was titrated by 0.1 M potassium hydroxide under an argon atmosphere. The solution was vigorously stirred during titration. Data was processed by HYPERQUAD (Gans et al. 1996).

^{13}C NMR at high pH values

Proton-decoupled Fourier transform ^{13}C NMR spectra were obtained on a Bruker Avance 400 spectrometer operating at 100.1 MHz for ^{13}C. Data was acquired in automation mode utilizing a broadband ATMA probe. A DSS solution in 10% deuterated water placed in a coaxial insert was used as an external reference. Spectra were obtained at room temperature (298.3 K) and no significant temperature variation was observed during the measurements.

Stock solutions of one of the carboxylic acids (citric, malic or lactic acid) were prepared to a concentration of 1.5 M. The pH value of the stock solutions was adjusted to 9.0 in order to ensure deprotonation of the carboxylic acid groups. The stock solutions were diluted in nine volumes of strong potassium hydroxide solution containing 10% of deuterated water. The pH value of the final solution was calculated from the hydroxide concentration. For pKa estimation, data of chemical shift from DSS as a function of pH was processed utilizing SPECFIT32 (Gampp et al. 1985).

Results

In order to confirm the ionisation of the hydroxyl group of citric acid at pH values lower than 12 (pKa = 11.6) (Migal and Sychen 1958) a potentiometric titration was carried out (Fig. 2a). Inspection of the high pH region, as depicted in Fig. 2b, demonstrates that citric acid does not possess any buffering capacity at pH values between 10.5 and 12, with the experimental line showing no significant difference from the trace representing a titration of a pure water sample. The dotted line represents the pH reduction as simulated by HYPERQUAD (Gans et al. 1996) for the existence of a species with a pKa value of 11.6 in solution, highlighting the inability of citric acid to buffer the solution against the addition of potassium hydroxide under these experimental conditions.

Being unable to detect a fourth pKa value below 12 for citric acid, we assessed the hydroxyl deprotonation at higher pH values. Due to the difficulties in measuring pH above 12 utilizing the glass pH electrode, ^{13}C NMR spectra of the different compounds were obtained in solutions with high concentrations of potassium hydroxide. Figure 3 shows the ^{13}C NMR spectra of citric acid (Fig. 3a) and the related α-hydroxycarboxylates malic acid (Fig. 3b) and lactic acid (Fig. 3c) in solutions at pH 10.5. Variance of the chemical shift relative to DSS with pH for the hydroxyl carbon of citric acid is represented in Fig. 4a. Plots of chemical shift as a function of pH for each carbon resonance of citric, malic and lactic acids are shown in Fig. 4b. The pH values of the different solutions were calculated from the known hydroxide concentration assuming that the buffering effect of the hydroxyl proton in the different compounds was negligible. The resulting curves differ from those published for the deprotonation of carboxylic acids in aqueous solution (Cistola et al. 1982; Quirt et al. 1974). While in the literature an increase in chemical shift is observed with deprotonation and the subsequent increase in the oxygen electron density and magnetic shielding, in this study decreases in the chemical shift were observed for all carbon resonance with the exception of the methylene groups in citric acid and the methyl group in lactic acid. Further, the observed chemical shift variations range from 0.04 to 0.16 ppm depending on the compound and carbon analysed while previous studies reported changes of several ppm units for the total deprotonation of carboxylic acids. These results indicate that only partial ionisation of the hydroxyl proton can be observed at pH values up to 14 with only the initial part of the titration curve being recorded. Addition of KCl (up to a 2 M final concentration) did not induce significant changes in chemical shifts, a result similar to that found for other aqueous carboxylic acids (Cistola et al. 1982)

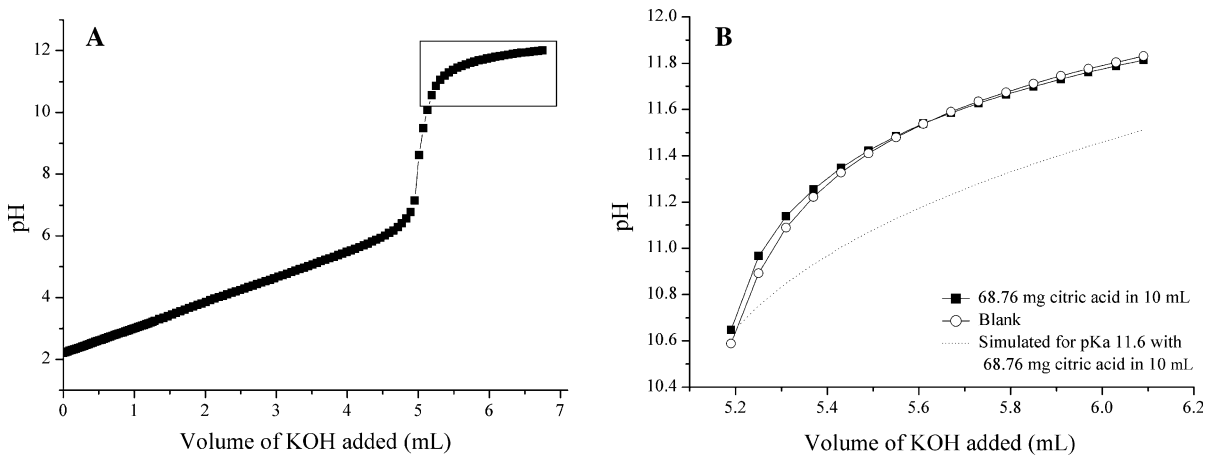

Fig. 2 Potentiometric titration of citric acid. **a** Potentiometric titration of citric acid (68.76 mg). **b** Overlap of the potentiometric titration data with blank at high pH range

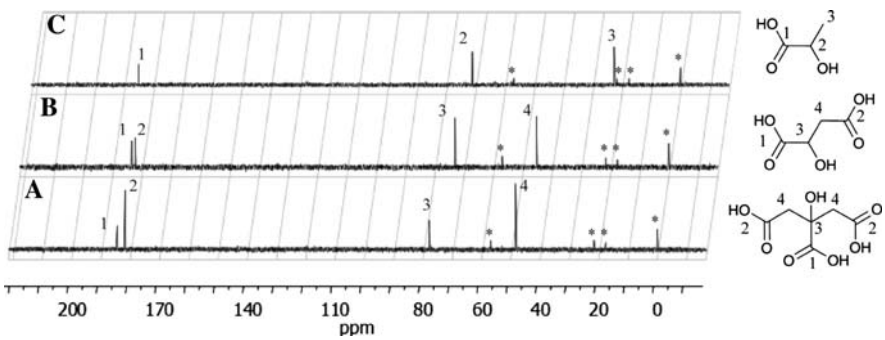

Fig. 3 ^{13}C NMR spectra of **a** citric acid, **b** malic acid and **c** lactic acid in aqueous solution with a pH value 10.5. NMR peaks marked with * arise from the DSS in the coaxial insert

reinforcing the interpretation that the chemical shift variation observed is due to the increase in the solution pH value.

Curve fitting with the software SPECFIT32 (Gampp et al. 1985) allowed the estimation of the pKa value for the hydroxyl group of the different α-hydroxycarboxylic acids (Table 1). For citric acid, data fitting for each individual carbon resonance was in good agreement. For malic and lactic acids only data for the carbon resonance where the maximum variance in chemical shift was higher than 0.05 ppm was used for the pKa estimation. This option was taken due to the difficulty in obtaining good curve fittings with lower variations and the elevated errors associated with such estimations. Reported standard deviations (SD) correspond to the highest SD value found when fitting data for individual carbons. The resulting pKa values (Table 1), confirmed the non existence of a fourth pKa value for citric acid below a solution pH = 14, but indicate that each of the three α-hydroxycarboxylate protons are more acidic than that of methanol (pKa = 15.5) (Volhardt and Schore 2003).

Discussion

Citric acid is a well established metal ligand of biological importance and has been demonstrated to form complexes with several metals of which iron assumes a particular relevance. The non-transferrin-bound iron (NTBI) pool in the sera of iron overloaded patients is shown to be bound to citrate (Grootveld et al. 1989). Further, citric acid plays a role in iron acquisition by bacteria and plants. In fact, evolution seems to have selected citrate as a selective Fe(III) ligand, as the chemical structure of many bacterial and fungi siderophores is based in that of citric acid (Drechsel and Winklemann 2000).

X-ray crystallography studies have shown that citric acid acts as a tridentate ligand (Matzapetakis

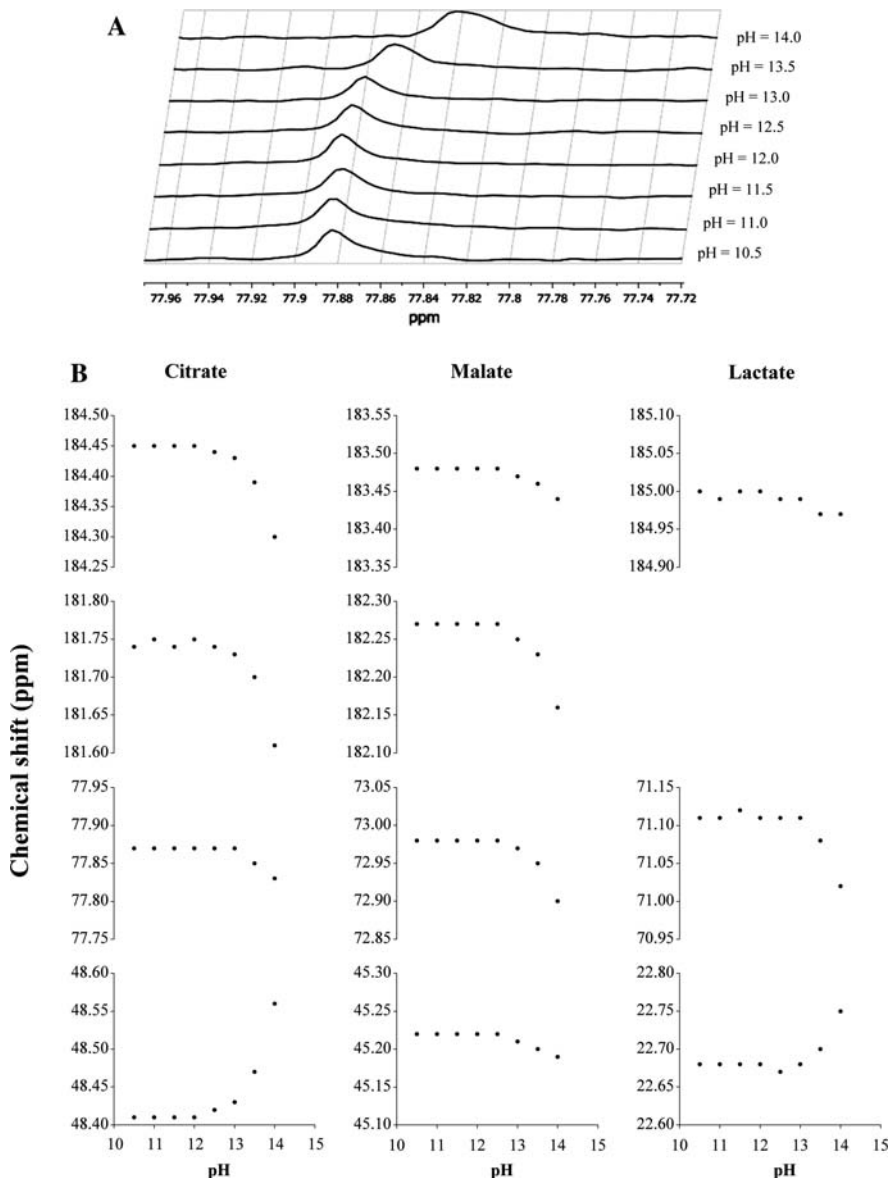

Fig. 4 Variation in chemical shift to DSS as a function of pH. **a** Influence of solution pH value on the chemical shift of the hydroxyl bound carbon resonance in citric acid. **b** Variations in chemical shift for all carbon resonance values as a function of the pH value for citric, malic and lactic acids

Table 1 pKa values for the hydroxyl group of citric, malic and lactic acids estimated from the variation of the carbon resonance chemical shifts with the solution pH value

	pKa (±SD)
Citric acid	14.4 (±0.3)
Malic acid	14.5 (±0.1)
Lactic acid	15.1 (±1.5)

SD, Standard deviation

et al. 2000b, 2001; Gautier-Luneau et al. 2005; Strouse et al. 1977) and contrary to what might be expected, coordination does not occur through the three carboxylate groups, but involves the α-hydroxy-carboxylate moiety and one of the two remaining carboxylates. As observed in the crystal structures, the hydroxyl group becomes deprotonated in complexes of strong Lewis acids such as Fe(III) and Al(III) (Matzapetakis et al. 1998, 1999), while deprotonation is not observed in complexes with divalent metals such as Fe(II) and Mn(II) (Matzapetakis et al. 2000b; Strouse et al. 1977). This being the case, it is necessary to determine a reliable value for the deprotonation constant of the alcohol group, in order to rigorously determine the stability constants

of citric acid metal complexes. One may assume that a similar relevance should be given to the hydroxyl pKa value of other α-hydroxycarboxylate chelators, as is the situation with several siderophores.

The number of structurally characterized siderophores which contain a α-hydroxycarboxylate moiety at the Fe(III) coordination site has been increasing rapidly. These compounds may be divided in two classes, one comprising siderophores derived from citric acid and the other, molecules which are unrelated to citrate but posses a α-hydroxycarboxylate group. In the case of rhizoferrin, staphyloferrin A and vibrioferrin the chelation unit from citrate is maintained, the derivatization occurring at the non-coordinating carboxylate group (Drechsel and Winklemann 2000). Forming a second subclass of citrate-derived siderophores are the so called "citrate-hydroxamates" such as rhizobactin 1021, aerobactin, acinetoferrin and the more recently characterized marine siderophores ochrobactins and synechobatins (Martinez and Butler 2007). The class of siderophores possessing a α-hydroxycarboxylate unit, not structurally related with citric acid, includes the hydroxylaspartate-containing ornibactins and aquachelins. Graminaceous plants, such as cereals and barley, when growing under iron deficient conditions produce α-hydroxycarboxylic acids, as typified by muginic acid (Drechsel and Winklemann 2000).

Despite the many variations found in siderophore structure, the fact that the α-hydroxycarboxylate group is so widespread in iron chelators produced by evolutionarily distant species, probably indicates that this structure is of particular relevance for Fe(III) coordination. In order to fully understand the chemical behaviour and the mechanism which led to the selection of this structural unit by nature, it becomes important to understand the ionisation behaviour of this moiety. For this reason, we studied the ionisation of the alcohol function of citric acid and the related compounds malic and lactic acids. To date, the only value reported in the literature for the pKa of the hydroxyl function of citric acid is 11.6 (Migal and Sychen 1958) and no values are known for malate and lactate. Carrano et al. (1996) found that the values for the protonation constants of the two hydroxyls in rhizoferrin are of the same order of magnitude (pKa = 10.05, pKa = 11.3) as the value reported for citrate. However, we failed to observe a fourth ionisation of citric acid at pH values up to 12 and so it was decided to investigate the behaviour of α-hydroxycarboxylates at higher pH values (up to 14) utilizing ^{13}C NMR. Despite finding significant changes to chemical shift for the carbon resonance when the pH value of the solution was equal or higher than 13, it became clear that total ionisation of any of the compounds studied does not occur at pH values lower than 14. The observed variations in chemical shift as a function of solution pH value showed that partial ionisation occurs and permitted the estimation of the pKa values of the alcohol function in the different acids studied. The hydroxyl group of citric and malic acids were found to have similar acidities [pKa = 14.4(±0.3) and pKa = 14.5(±0.1), respectively], with that of lactic acid being less acidic [pKa = 15.1(±1.5)]. The estimated pKa values are lower than the values reported for simple aliphatic alcohols such as methanol and ethanol. This increase in acidity may be explained by the electron withdrawing inductive effect of the α-carboxylate group. Carboxylate groups are well characterized electron acceptor groups through π-bound conjugation, but the presence of two oxygen atoms with higher electronegativity than the carbon atoms is also expected to result in an electron withdrawing inductive effect. In the studied compounds the conjugation effect with carbon–carbon double bounds is absent, but the inductive effect of the α-carboxylate group is responsible for a decreased electron density in the alcohol function and the corresponding lower pKa value. The presence of the β-carboxylate groups in citric and malic acids explains the enhanced reduction of the pKa value when compared to lactic acid. Further, the electron donating inductive effect of the methyl group of lactic acid counter balances the effect of the carboxylate function.

Interestingly, when investigating the ionisation behaviour of rhizoferrin, we confirmed the values reported in the literature (Carrano et al. 1996). The increased acidity of the alcohol groups in this siderophore may be explained by the influence of intra-molecular hydrogen bonding. As noted by Sykes 1986, the effect of intra-molecular hydrogen bonding should not be disregarded in the energy stabilization of molecules. In rhizoferrin, two sets of intra-molecular hydrogen bounds are possible (Fig. 5a). The hydroxyl groups may form hydrogen bonds with both the deprotonated carboxylate in the citric acid moiety the amide hydrogen atom. This latter bond may also form with the hydroxyl anion

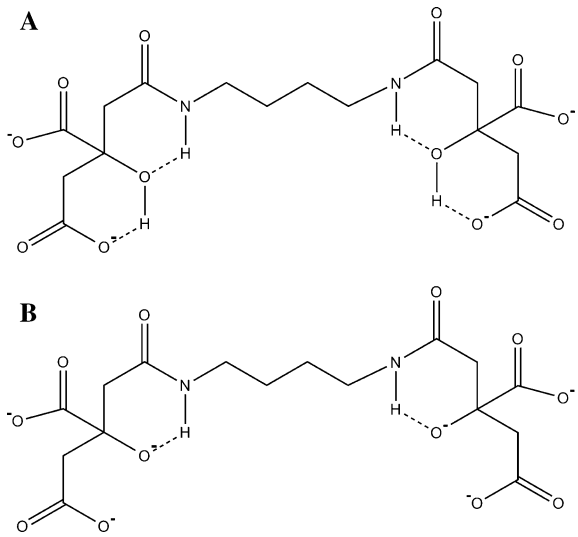

Fig. 5 Schematic representation of intra-molecular hydrogen bonding likely to occur in **a** $[rhizoferrin]^{4-}$ and **b** $[rhizoferrin]^{6-}$

formed upon the alcohol deprotonation (Fig. 5b). In cases where intra-molecular hydrogen bounding occours in both in the undissociated acid and the conjugate anion, the dissociation of the hydroxyl function will be facilitated (Sykes 1986). Such intra-molecular hydrogen bonding in rhizoferrin would stabilize the formed hydroxy anion, explaining the greater decrease in the pKa values. No such enhancement is possible with citric acid as all the carboxylate functions will be fully deprotonated at neutral to alkaline pH values.

The chemistry of α-hydroxycarboxylic acids is complex. The pKa value for citrate found in the current study (14.4) is appreciably higher than the literature value (11.6) and this unfavourable energy of deprotonation should be considered when calculating stability constants of trivalent metal–citrate complexes.

Acknowledgments Silva A. M. N. would like to acknowledge "Fundacao para a Ciencia e Tecnologia", Lisboa Portugal for his PhD grant [SFRH/BD/22633/2005]. The authors would like to thank Jane Hawkes for assistance with NMR spectroscopy.

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