Table I. Kinetic Data for the Isomerization of γ-$[Ru(Azpy)_2Cl_2]$ to the β Structure (25 °C)[a]

$[OH^-]$, M	$10^4 k_{obsd}$, s^{-1}
0.0060	3.1 ± 0.2
0.012	5.8 ± 0.1
0.024	8.9 ± 0.7

[a] In 1:1 methylene chloride:ethanol solution. [Complex] = 1.50×10^{-4} M.

not attempted a correlation between solvent differences and varying isomerization rates but have qualitatively observed some differences between various water–ethanol mixtures. Chakravorty's group[5] has noted boiling aqueous sodium hydroxide to react with all three isomers of $[Ru(Azpy)_2Cl_2]$ to yield isomeric mixtures of diaquo species (after acidification). This could be due to the excess hydroxide; we find such conditions to indeed lead to mixtures, probably because of the formation of $[Ru(Azpy)_2(OH)_2]$, which is slower to isomerize (above) than the chlorohydroxo intermediates otherwise formed.

Highly stereospecific reactions such as these should be helpful in elucidating reaction mechanisms. While many base-catalyzed reactions have been concluded to involve conjugate base mechanisms,[14] such cannot be the case here where there are no ionizable protons. We presume these isomerizations to involve nucleophilic attack; this seems reasonable since the isomerization rate is markedly dependent on the presence of base. We have not investigated a range of nucleophiles but have found chloride ion, in the absence of base, to be ineffective. However, Chakravorty's group have found tertiary phosphines to be effective nucleophiles for the simultaneous substitution and isomerization of γ-$[Ru(Azpy)_2Cl_2]$.[4] Their observation of the rate dependence on entering ligand concentration makes nucleophilic attack a plausible mechanism.

We have examined the kinetics of the hydroxide ion reaction with the γ isomer at 25 °C. Under pseudo-first-order conditions linear plots were obtained. As the hydroxide concentration was varied different first order rate constants resulted (Table I) demonstrating the base dependence. However, the differences in k_{obsd} are not linear with base. This stands in contrast to the second-order dependence observed by Chakravorty's group in the reaction of the gamma isomer with phosphines.[4]

Rate dependence on entering ligand concentration does not require an associative reaction, as demonstrated by Allen and Ford.[15] However, we feel all of our observations are consistent with a two-step process

$$[Ru(Azpy)_2Cl_2] + OH^- \xrightarrow{k_2} [Ru(Azpy)_2Cl_2](OH)^-$$

$$[Ru(Azpy)_2Cl_2](OH)^- \xrightarrow{k_1} [Ru(Azpy)_2Cl(OH)] + Cl^-$$

or

$$[Ru(Azpy)_2Cl_2] \xrightarrow{k_1} [Ru(Azpy)_2Cl]^+ + Cl^-$$

$$[Ru(Azpy)_2Cl]^+ + OH^- \xrightarrow{k_2} [Ru(Azpy)_2Cl(OH)]$$

While one step is hydroxide ion dependent the other is not. Thus, the overall reaction does not necessarily show a linear dependence on hydroxide ion. We observe no isosbestic points in the spectra during the course of the reaction; this is consistent with consecutive reactions.[16]

Formation of the β structure could occur through either a dissociative or associative path, the dissociative path requiring a trigonal-bipyramidal intermediate. For an associative path it should be noted that in going from the trans γ structure to the cis β form both ends of one chelating ligand must move with respect to the other. If one octahedral face containing a chloride and one chelating Azpy simply rotates the β structure is produced. Hydroxide ion, in the process of displacing a chloride, must cause this rotation.

Registry No. *trans*-γ-$[Ru(Azpy)_2Cl_2]$, 73952-48-4; *cis*-β-$[Ru(Azpy)_2Cl_2]$, 74006-30-7; *cis*-α-$[Ru(Azpy)_2Cl_2]$, 84027-71-4; OH^-, 14280-30-9.

(14) Basolo, F.; Pearson, R. G. *Mechanisms of Inorganic Reactions*; Wiley: New York, 1967.
(15) Allen, R. J.; Ford, P. C. *Inorg. Chem.* **1972**, *11*, 679.
(16) Wilkins, R. G. *The Study of Kinetics and Mechanism of Reactions of Transition Metal Complexes*; Allyn and Bacon: Boston, MA, 1974; p 22.

Contribution from the Departments of Chemistry, University of Florence, Florence, Italy, and University of Modena, Modena, Italy

Evidence of a Metal–Synergistic Anion Bond in Thallium(III) Transferrin

I. Bertini,*† L. Messori,† G. C. Pellacani,‡ and M. Sola‡

Received June 10, 1987

Transferrins are a class of proteins that bind many metal ions, particularly iron(III);[1] the affinity for metal ions is drastically enhanced by the presence of bicarbonate, which therefore is called the synergistic anion. A tight ternary complex between the protein, the metal ion, and the synergistic anion forms in both metal binding sites. The lack of any ^{13}C NMR signal in Fe^{III}_2–Tf–$(^{13}CO_3)_2$ (Tf = human serum transferrin) is consistent with direct binding of carbonate to the paramagnetic iron(III) ion.[2] The same indication follows from the spectral variation dependence on the nature of the synergistic anion, e.g. carbonate, oxalate, etc.[3-5] The ^{13}C NMR spectra of several diamagnetic metallotransferrins with carbonate or oxalate as the synergistic anion have been interpreted[6,7] in terms of the synergistic anion bridging the metal and a positively charged group as previously proposed ("interlocking sites model");[8] this arrangement should contribute to the stability of a conformation suitable for metal coordination. The X-ray structure, which is now available for iron lactoferrin at 3.2-Å resolution,[9] is consistent with the above model and suggests that Arg 121 (477 in the C-terminal lobe) and the N-terminus of the α-helix 121–137 (477–492 in the C-terminal lobe) are the sites to which the synergistic anion is linked. However, the conclusive spectroscopic evidence of a direct bond between the synergistic anion and the metal may arise from the observation of the coupling between two magnetic nuclei, one on the synergistic anion and the other the metal nucleus itself. For this purpose we have investigated ^{13}C and ^{205}Tl NMR spectra of the system Tl^{III}_2–Tf–$(^{13}CO_3)_2$. The investigation of this system without enriched carbonate was reported by us some time ago.[10] The experimental procedures were the same as previously reported.[7,10]

The ^{13}C NMR spectrum of Tl^{III}_2–Tf–$(^{13}CO_3)_2$ (90%-enriched bicarbonate) in 0.1 M Tris buffer, pH 8.3, in the 190–150 ppm region from TMS, measured at 75.4 MHz, is reported in Figure 1A. Such a spectrum shows, besides natural-abundance protein signals and the free bicarbonate signal (a), three more signals respectively located at 168.6 (b), 168.3 (c), and 164.7 ppm (d), with relative intensities 1:1:2. A more simplified spectral pattern is obtained in the case of the $Fe_C Tl_N$–Tf–$(^{13}CO_3)_2$ derivative with thallium(III) specifically loaded in the N-terminal site, at the same pH. This derivative could be easily prepared by reacting apotransferrin at pH 5.5 with 1 equiv of iron(III) nitrate, raising then the pH to 8.3 and adding 1 equiv of thallium(III) chloride. Indeed the ^{13}C NMR spectrum of the latter derivative shows only two signals of comparable intensity for the protein-bound synergistic anion, respectively located at 168.6 and 164.7 ppm (Figure 1B).

† University of Florence.
‡ University of Modena.

0020-1669/88/1327-0761$01.50/0

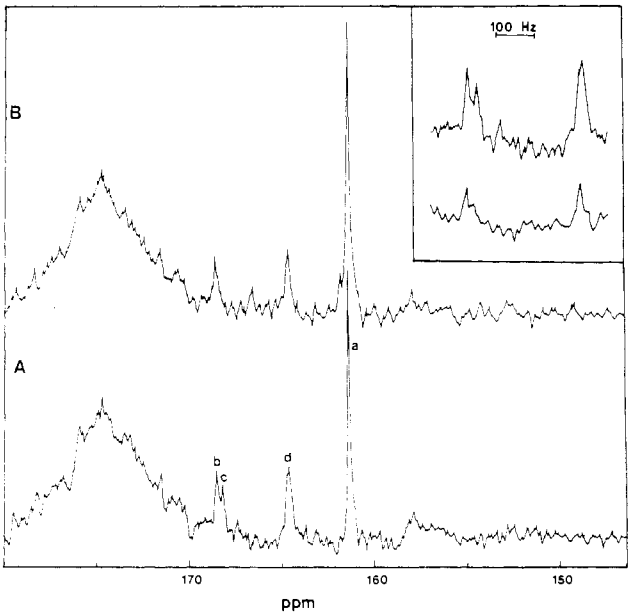

Figure 1. Proton-decoupled 75.4-MHz ^{13}C NMR spectra of the carbonyl region of thallium(III) transferrin derivatives in the presence of a 2-fold excess of ^{13}C-enriched sodium bicarbonate (protein concentration 1.5 mM, Tris-HCl 0.1 M, pH 8.3): (A) Tl_2-Tf-($^{13}CO_3$); (B) Fe_CTl_N-Tf-$(^{13}CO_3)_2$. The inset shows the detail of the signals typical of the bound anion, obtained at both 75.4 MHz (upper) and 50.3 MHz (lower), reported on a hertz scale. Spectra were obtained with a 50° flip angle, 2-s pulse delay, 10-kHz spectral width, and 15000 scans. Quadrature detection was employed, and a line broadening of 6 Hz was applied. T = 300 K.

Table I. ^{13}C NMR Parameters of Bound Carbonate in Thallium(III) Transferrin Derivatives

compd	chem shift, ppm		$J(^{13}C-^{205}Tl)$, Hz	
	N site	C site	N site	C site
Tl_2-Tf-$(^{13}CO_3)_2$	166.64	166.49	290	265
Tl_NFe_C-Tf-$(^{13}CO_3)_2$	166.64		290	

These results are quite different from those found in the case of the analogous Al_2-Tf-$(^{13}CO_3)_2$ and Ga_2-Tf-$(^{13}CO_3)_2$ derivatives, which exhibited only one signal for the specifically bound synergistic anion, located around 166 ppm.[6,7] The best explanation for the observed spectral pattern is that the ^{13}C nucleus of the synergistic anion is strongly magnetically coupled to the ^{205}Tl nucleus so that its ^{13}C signal is split into a doublet. So, the ^{13}C NMR spectrum of Tl_2-Tf-$(^{13}CO_3)_2$ would originate from the superposition of two doublets; discrimination between them is obtained straightforwardly from the spectrum of monothallium monoferric transferrin. The ^{13}C NMR spectral parameters for both sites are reported in Table I. From inspection of the table it appears that the synergistic anion in the two sites exhibits very similar but not equivalent chemical shifts and $^{205}Tl-^{13}C$ coupling constant values; the former values closely resemble those found for Al_2-Tf-$(^{13}CO_3)_2$ and Ga_2-Tf-$(^{13}CO_3)_2$. These findings are consistent with the view that the two metal sites in transferrins are very similar but not identical.

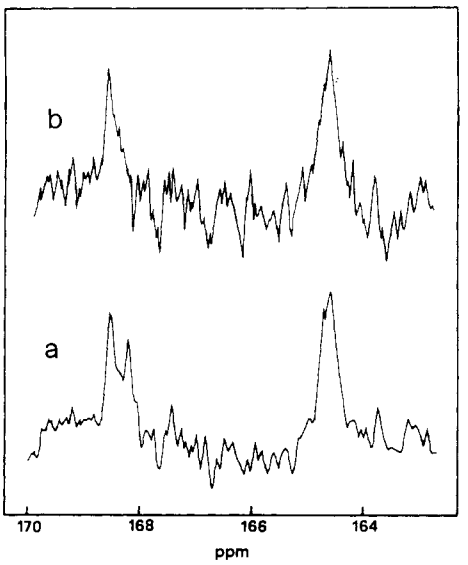

Figure 2. Effect of the addition of excess ^{12}C bicarbonate on the ^{13}C NMR spectrum of Tl_2-Tf-$(^{13}CO_3)_2$. The figure shows the 75.4-MHz ^{13}C NMR signals typical of the bound carbonate as they appear (a) in absence or (b) in presence of 0.1 M natural-abundance sodium bicarbonate. The latter spectrum was run in the first hour after bicarbonate addition. The experimental conditions were the same as in Figure 1.

As expected and as a confirmation of the assignment, we noted that the splitting in hertz of the ^{13}C NMR signals is independent of the magnetic field; this could be ascertained by recording the same spectra at 50.3 MHz (Figure 1, inset). The extent of the coupling constants (290 and 265 Hz) is typical of a $^2J(^{205}Tl-^{13}C)$ coupling as it results from comparison with literature values;[11] this represents conclusive evidence of direct binding of the carbonate anion to the metal in transferrins.

We have also attempted to observe the $^{205}Tl-^{13}C$ coupling in the ^{205}Tl NMR spectra of the dithallium derivative. Unfortunately, in this case, no clear splitting could be observed owing to the intrinsic broadness of the signals and to an unfavorable signal to noise ratio.

The ^{13}C NMR signals were also monitored in the presence of increasing amounts of perchlorate up to 1 M concentration since such an anion is reported to affect both the conformational state of the metal sites and the metal release kinetics.[1] Indeed, no major changes in the spectra could be detected even under drastic conditions.

Finally, the exchange between bound ^{13}C-enriched carbonate and excess ^{12}C bicarbonate was investigated: a spectrum recorded on a Tl^{III}_2-Tf-$(^{13}CO_3)_2$ derivative during the first hour after addition of 0.1 M ^{12}C bicarbonate, at room temperature, indicated that the exchange had occurred almost completely at the C-terminal site and partially also at the N-terminal site (Figure 2). This behavior is consistent with previously reported data on the exchange kinetics of the synergistic anion in iron(III) and cobalt(III) transferrin[6,12] (i.e., the C-terminal site is the faster exchanging site) although the overall process is faster in the case of thallium(III) derivatives.

Acknowledgment. We wish to thank Prof. Brian Mann from the University of Sheffield for illuminating discussion and suggestions.

Registry No. Tl, 7440-28-0; CO_3^{2-}, 3812-32-6.

(1) Chasteen, N. D. *Adv. Inorg. Biochem.* **1983**, *5*, 201.
(2) Harris, D. C.; Gray, G. A.; Aisen, P. *J. Biol. Chem.* **1974**, *249*, 5261.
(3) Aisen, P.; Pinkowitz, R. A.; Leibman, A. *Ann. N.Y. Acad. Sci.* **1973**, 337.
(4) Campbell, R. F.; Chasteen, N. D. *J. Biol. Chem.* **1977**, *252*, 5996.
(5) Bertini, I.; Luchinat, C.; Messori, L.; Monnanni, R.; Scozzafava, A. *J. Biol. Chem.* **1986**, *261*, 1139.
(6) Zweier, J. L.; Wooten, J. B.; Cohen, J. S. *Biochemistry* **1981**, *20*, 3505.
(7) Bertini, I.; Luchinat, C.; Messori, L.; Scozzafava, A.; Pellacani, G.; Sola, M. *Inorg. Chem.* **1986**, *25*, 1782.
(8) Schlabach, M. R.; Bates, G. W. *J. Biol. Chem.* **1975**, *250*, 2177. Bates, G. W.; Schlabach, M. R. *J. Biol. Chem.* **1975**, *250*, 2182.
(9) Anderson, B. F.; Baker, H. M.; Dodson, E. J.; Norris, G. E.; Rumball, S. V.; Waters, J. M.; Baker, E. N. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 1769.
(10) Bertini, I.; Luchinat, C.; Messori, L. *J. Am. Chem. Soc.* **1983**, *105*, 1347.
(11) Hinton, J. F.; Metz, K. R.; Briggs, R. W. In *Annual Reports on NMR Spectroscopy*; Webb, G. A., Ed.; Academic: New York, 1982; Vol. 13, pp 211–318.
(12) Aisen, P.; Leibman, A., Pinkowitz, R. A.; Pollack, S. *Biochemistry* **1973**, *12*, 3679.