A simple carbonic anhydrase model which achieves catalytic hydrolysis by the formation of an 'enzyme–substrate'-like complex †

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One of the attractive features of biomimetic catalysts is their amenability, relative to their enzyme counterparts, to the testing of structural and mechanistic hypotheses. Thus, the reproduction of the active site of an enzyme in a model system provides a tool which is free of much of the complexity of the enzyme. Using this approach, we describe a new model of the active site of the hydrolytic enzyme carbonic anhydrase (CA). The model (**1**) is composed of a Zn^{2+} complex of the tripodal ligand 1,1,1-tris(aminomethyl)ethane. We find that, in analogy to CA, the complex possesses a water molecule whose pK_a is reduced to 8.0 by coordination to the chelated Zn^{2+} ion. We demonstrate that the complex catalyses the hydrolysis of a model ester substrate (*p*-nitrophenyl acetate, *p*-NPA) with a second-order rate constant (k_2) of 0.71 M^{-1} s^{-1} (55.0 °C, pH 8.20, ionic strength, $I = 0.1$ M, aqueous solution), and moreover that it does so with Michaelis–Menten kinetic behaviour ($K_m = 7.6$ mM; 45.0 °C, pH 8.20, $I = 0.1$ M, 50% v/v CH_3CN–H_2O). The comparison of these data with those for CA suggests that the hydrophobic cavity and Thr199 residue (which lie adjacent to the active-site of the enzyme) contribute only marginally to the pK_a reduction of the Zn^{2+}-bound water molecule. Despite the absence of these moieties, the chelated Zn^{2+} ion is still capable of forming an 'enzyme–substrate'-like complex, but the stability of the complex is approximately one order of magnitude smaller than that of the enzyme.

Introduction

Carbonic anhydrase[1] (CA) is a prominent member of the family of Zn^{2+} hydrolase enzymes, which are widely-employed in nature to catalyse the hydrolysis and hydration of a variety of substrates.[2] It has the highest turnover number of all known enzymes (1.4×10^6 s^{-1} at 25 °C for human CA isozyme II),[1a] and as such approaches the limits of diffusion control and enzymatic perfection. The key to the hydrolytic activity of the enzyme is a Zn^{2+} ion, which is chelated in a tetrahedral array by three histidine residues and a water molecule (Fig. 1). The Lewis acidity of the chelated Zn^{2+} ion serves to lower the pK_a of the bound water to *ca.* 7.5, furnishing an enhanced concentration of hydroxide ions at the physiological pH.[1,3] It is widely-believed that carbonic anhydrase carries out the efficient hydration of carbon dioxide to hydrogen carbonate by the nucleophilic attack of the Zn^{2+}-bound hydroxide ion on the substrate.[1]

The mechanism is outlined in greater detail in Scheme 1. The coordinated hydroxide ion is stabilised by hydrogen-bonding with a neighbouring threonine residue (Thr199), which is itself activated by Glu106. The whole array is encased within a 17 Å-deep cavity, the walls of which are lined with hydrophobic residues, which are particularly prevalent in the region of the 'deep' water. The substrate displaces the 'deep' water, thus positioning itself closely to the zinc-bound hydroxide ion. The hydration reaction is facilitated by the proximate orientation of the Zn^{2+}-bound hydroxide ion to the enzyme–substrate complex, which is very short-lived and therefore cannot be observed directly (although its presence is inferred by the observation of

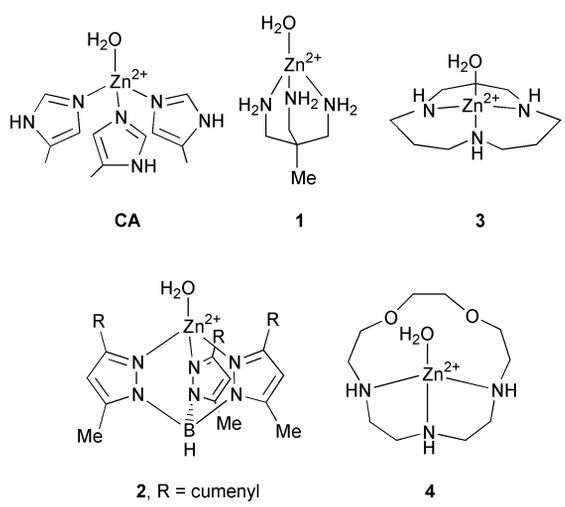

Fig. 1

Michaelis–Menten kinetic behaviour). The HCO_3^- produced diffuses from the pocket, and is replaced by a molecule of water. The Zn^{2+} ion binds a further molecule of water, which is deprotonated in the final, rate-limiting, step of the catalytic cycle, the lability of the Zn^{2+} ion facilitating the Zn^{2+}–H_2O ↔ Zn^{2+}–OH^- ↔ Zn^{2+} interconversion.[4] The enzyme is clearly tailored for the catalysis of the CO_2–HCO_3^- interconversion, but nonetheless it demonstrates considerable promiscuity in the accommodation of other substrates. Thus, CA catalyses the hydrolysis of carboxylic§ and carbonate esters, acid

† Electronic supplementary information (ESI) available: experimental data. See http://www.rsc.org/suppdata/p2/b1/b107683n/

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§ Including *p*-nitrophenyl acetate, which is widely-used in kinetic studies of CA because the *p*-nitrophenolate anion produced at pH > 7 is strongly chromophoric ($\lambda_{max} \approx 405$ nm, $\varepsilon = 2.1 \times 10^4$ M^{-1} cm^{-1}), and the rate of reaction is such that the use of stopped-flow techniques is not necessary.

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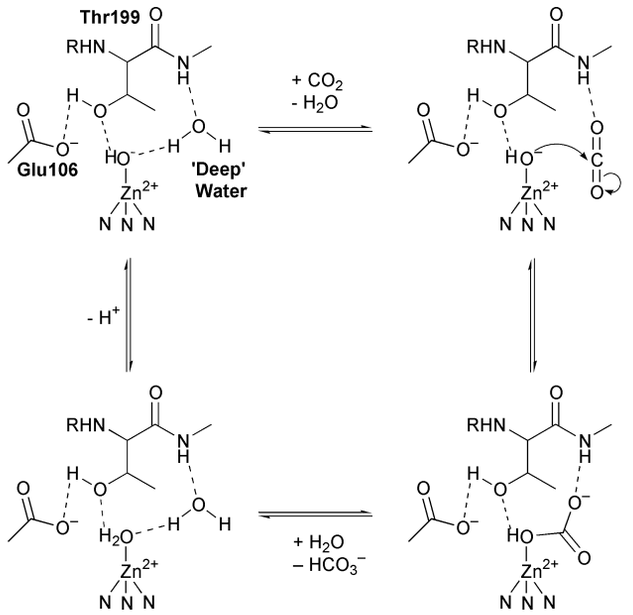


Scheme 1 Proposed mechanism for the hydration of CO_2 by carbonic anhydrase.

chlorides, sulfonate esters, sulfonyl chlorides and dialkyl monoaryl phosphate esters, and the hydration of aldehydes and pyruvic acid.[5]

In order to explore the active-site 'core' required for the activity of CA, several excellent model systems have been described (Fig. 1). In addition to structural models, which demonstrate the facility with which Zn^{2+} forms tetrahedral complexes composed of three nitrogen atoms and an apical water molecule or hydroxide ion,[6] the hydrolytically-active model (**2**) reported by Ruf and Vahrenkamp cleaves activated esters in hydrophobic solvents such as benzene and chloroform, albeit in a stoichiometric reaction.[7] Other model complexes have been shown to possess CA-like activity in an aqueous or semi-aqueous environment.[8] Perhaps the example most faithful to CA is that described by Kimura *et al.*, which consists of a Zn^{2+} complex (**3**) of the macrocyclic polyamine ('azacrown') 1,5,9-triazacyclododecane.[9] The complex was shown to catalyse the hydration of acetaldehyde and the hydrolysis of methyl acetate, the pH–rate profile of the latter reaction being sigmoidal in shape, with a point of inflection at pH 7.3, corresponding to the pK_a of the Zn^{2+}-bound hydroxide ion. Another complex, **4**, reported recently by Bazzicalupi *et al.* possesses a polyether chain which partially models the hydrophobicity of the enzyme active site.[10] The model catalyses the hydrolysis of *p*-nitrophenyl acetate (*p*-NPA) with a sigmoidal pH–rate profile whose point of inflection corresponds to the pK_a of the Zn^{2+}-bound hydroxide ion (8.8) which was suggested by potentiometric titration.

These, and other[11] examples illustrate that simple model systems are capable of mimicking the behaviour of large, complex enzymes remarkably well. The results reported so far support the proposed mechanism of CA, but Michaelis–Menten kinetic behaviour (a key aspect of enzyme-catalysed reactions)[12] has not been reproduced in a model system. Consequently, the extent to which the hydrophobic cavity and Thr199 contribute to the formation of the 'enzyme–substrate' complex has been less well understood. Herein, we report that the Zn^{2+} complex (**1**) of the tripodal ligand 1,1,1-tris(aminomethyl)ethane is a good model of CA. The complex catalyses the hydrolysis of the ester *p*-NPA at approximately physiological pH and does so with Michaelis–Menten kinetic behaviour, the magnitude of which suggests that a relatively 'naked', chelated Zn^{2+} ion is itself capable of forming an 'enzyme–substrate'-like complex, albeit with a stability which is about 13 times less than that of the enzyme.

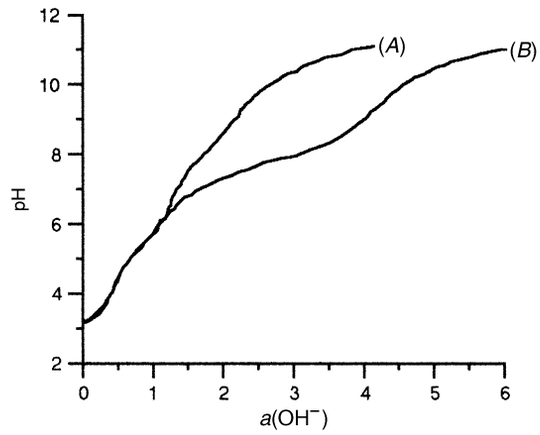


Fig. 2 Plots obtained for the titration of (*A*) tris(aminomethyl)ethane·3$HClO_4$ (1 mM) and (*B*) tris(aminomethyl)ethane·3$HClO_4$ (1 mM) + Zn^{2+} (1 mM) against NaOH (0.1 M). (25 ± 0.1 °C, aqueous, I = 0.1 M).

Results and discussion

The characterisation of complex 1

^{1}H NMR. A solution of complex **1** (1 : 1 tris(aminomethyl)ethane–Zn^{2+}) suitable for NMR analysis was prepared as described in the Experimental section. The ^{1}H NMR spectrum (recorded at pH 8.8 in 20% v/v D_2O–CD_3CN) indicates the presence of predominantly (>95%) one species. Three pronounced signals are observed, *viz.* the apical methyl protons at 0.57 ppm (0.83 ppm in the absence of Zn^{2+}), the methylene protons at 2.97 ppm (2.58 ppm in the absence of Zn^{2+}) and the amino protons at 2.77 ppm (not observed in the absence of Zn^{2+}). The simplicity of the spectrum indicates that a single, strong complex is formed. The alternative explanation (time-averaged signals due to multiple complexes which interchange rapidly on the NMR timescale) is discounted by the observation of distinct NH_2 and *H*OD signals, since the rate of proton exchange between NH_2 and HOD would be expected to be greater than the rate of complex interconversion.

Potentiometric titration. Having established that Zn^{2+} and tris(aminomethyl)ethane form essentially one stable complex, potentiometric titrations were performed to explore the nature of the Zn^{2+}-binding moieties. Plots (Fig. 2) were obtained for the titration of a solution of tris(aminomethyl)ethane·3$HClO_4$ (*A*), and a solution of tris(aminomethyl)ethane·3$HClO_4$ containing one equivalent of $Zn(ClO_4)_2$ (*B*), against 0.1 M sodium hydroxide (expressed as equivalents of OH^-, *a*). Plot (*A*) clearly shows three points of inflection corresponding to the sequential deprotonation of the nitrogen atoms of the ligand. The pK_a values for tris(aminomethyl)ethane (pK_a^1 = 10.4, pK_a^2 = 8.6, pK_a^3 = 5.8) which are indicated by the plot are in agreement with literature values.[13] Plot (*B*) indicates that an additional equivalent of OH^- is consumed in the presence of Zn^{2+}. Of the four pK_a values, the lowest two correspond almost exactly to those of (*A*), and are attributed to the deprotonation of the two least basic nitrogen atoms. The pK_a of the most basic nitrogen atom is reduced from 10.4 to 7.3, reflecting the complete chelation of the Zn^{2+} ion by the three donors. The fourth equivalent of OH^- corresponds to pK_a = 8.0, and is attributed to the deprotonation of a Zn^{2+}-bound water molecule, suggesting that complex **1** could function as a CA-like hydrolytic catalyst with a rate optimum above pH ≈ 8.

The apparent pK_a (8.0) is not as close to that of the enzyme (7.5) as is that in the case of **3** (7.3), but is closer than the corresponding values for $Zn(H_2O)_6^{2+}$ (9.6)[14] and **4** (8.8). The amine donors of tris(aminomethyl)ethane are not held in the relatively rigid macrocyclic arrangement of those belonging to the ligand in **3**, but they are free to adopt a similar tripodal

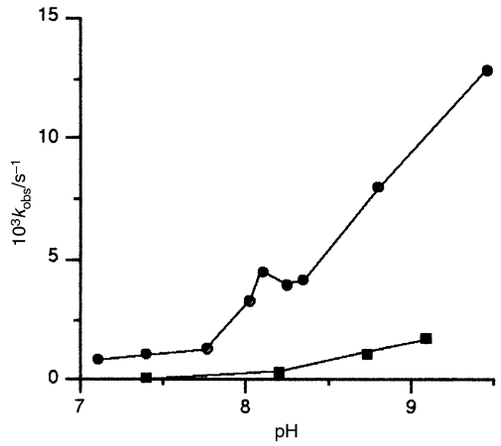


Fig. 3 pH–rate profile for the liberation of *p*-nitrophenolate from *p*-NPA in the presence of **1** (1 mM) + buffer (50 mM) (●), and in the presence of buffer only (50 mM) (■). ([*p*-NPA] = 20 μM, 55 ± 0.1 °C, aqueous, buffer = MES, MOPS or CHES, I = 0.1 M.)

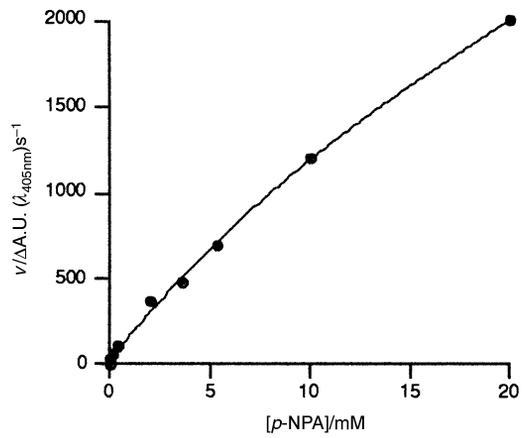


Fig. 4 Michaelis-Menten plot for the liberation of *p*-nitrophenolate from *p*-NPA by **1** (50 μM). (pH 8.20, 45 ± 0.1 °C, 50% v/v CH_3CN–H_2O, 50 mM MOPS buffer, I = 0.1 M.) ΔA.U. = ΔAbsorbance (arbitrary units).

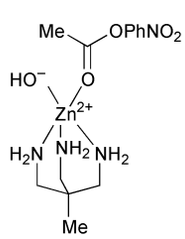
configuration, and this is particularly likely in the presence of a species (such as a proton or Zn^{2+} ion) to which they can chelate. This is manifested by the distribution of the pK_a values of tris(aminomethyl)ethane over a wide pH range, in common with macrocyclic polyamines (such as the ligand alone in **3**), in which the amine donors 'cooperate' to bind a single proton. In contrast, the relatively high pK_a of the Zn^{2+}-bound water molecule in **4** may arise from the non-tripodal nitrogen donor array of the parent ligand. We conclude that the hydrophobic cavity and Thr199 of CA do not greatly influence the pK_a of the Zn^{2+}-bound water molecule, and that this is more significantly affected by the relative geometries of the three donor nitrogen atoms.[14–16]

Complex 1 as a catalyst for ester hydrolysis

Catalysis as a function of pH. A pH–rate study was conducted, in order to determine whether complex **1** catalyses ester hydrolysis as anticipated. The pH–rate profile for the hydrolysis of *p*-NPA (observed as the release of the *p*-nitrophenolate anion) in the presence of complex **1** is reproduced in Fig. 3, together with the profile obtained for buffer alone in the absence of complex **1**. (The reactions were run under pseudo first-order conditions, with the catalyst in excess.) It is clear from the plots that complex **1** catalyses the hydrolysis of *p*-NPA throughout the pH range studied, with a marked increase in the rate at pH 8. The rate enhancement afforded by the catalyst (with respect to buffer alone) is 11.5 at pH 8.2. The pH (8.0) at which the sharp increase in rate occurs corresponds to the pK_a of the Zn^{2+}-bound water molecule which was suggested by the titrimetric data (Fig. 2). It is therefore reasonable to propose that the sharp increase in rate arises from the nucleophilic attack of a Zn^{2+}-bound hydroxide ion on the substrate, supporting the proposed mode of action of CA.

Michaelis–Menten kinetics. Michaelis–Menten saturation kinetics is observed during the hydrolysis of *p*-NPA by complex **1**. Thus, a plot of log v (where v is the initial rate of reaction) against [*p*-NPA] (Fig. 4) indicates the saturation of the catalyst by the substrate. Replotting of the data in Lineweaver–Burk form (not shown) gives a value of K_m = 7.6 mM (45.0 °C, pH 8.20, I = 0.1 M, 50% v/v CH_3CN–H_2O). To our knowledge, this is the first demonstration of Michaelis–Menten kinetics in a carbonic anhydrase model, and the absence from complex **1** of the hydrophobic cavity and substrate-binding residue (Thr199) of CA enables an estimation of their role in catalysis to be made. The value of K_m for the hydrolysis of *p*-NPA by complex **1** is approximately 13 times greater than that of carbonic anhydrase[17] (K_m = 0.57 mM), and the hydrophobic cavity and/or Thr199 residue therefore appear to increase the stability of the 'enzyme–substrate' complex, leading to a reduction in the value of K_m. This finding is in agreement with a study in which site-directed mutagenesis was used to alter the nature of the hydrophobic cavity of the enzyme itself.[18]

The most plausible substrate-binding moiety for the formation of the 'enzyme–substrate'-like complex with **1** is the Zn^{2+} ion itself. The binding of the carbonyl oxygen atom of the substrate to the Zn^{2+} ion would position the carbonyl carbon atom of the substrate immediately adjacent to the nucleophilic hydroxide ion. The attack of the hydroxide ion on the substrate would thus be highly-favoured, since this would approximate to an intramolecular reaction. The formation of a five-coordinate 'enzyme–substrate'-like complex is consistent with the well-established amenability of Zn^{2+} to changes in coordination number.[14,19] The demonstration of an 'enzyme–substrate'-like complex using the simple model **1** therefore suggests that, important as the nature of the environment surrounding the CA Zn^{2+} ion appears to be in forming the 'enzyme–substrate' complex, a relatively 'naked' Zn^{2+} complex is itself sufficient to bind a substrate molecule with substantial affinity. The elaborately-functionalised active-site of CA has presumably evolved to afford a degree of substrate specificity and to facilitate the formation of the 'enzyme–substrate' complex. A biomimetic analogy to this is the demonstration by Zhang and Breslow that a tailored, highly hydrophobic ester forms a relatively strong complex with a Cu^{2+}-based 'artificial hydrolase' equipped with appropriately-positioned hydrophobic cavities.[20]



'Enzyme-substrate'-like complex

Catalytic efficiency. The rate of hydrolysis of *p*-NPA as a function of [**1**] is depicted in Fig. 5 as a plot of k_{obs} against [**1**]. The plot is essentially linear with a slope (r^2 = 0.993, equating to the second-order rate constant, k_2) of 0.71 M^{-1} s^{-1} (55.0 °C, pH 8.20, I = 0.1 M) in the region [**1**] ≤ 3 mM. The slope of the plot approximates to zero at [**1**] ≥ 3 mM, which is accounted for by the lack of solubility of **1** at >3 mM. The corresponding value for CA is 97 M^{-1} s^{-1} (25 °C, pH 7.20, I = 0.09 M),[17] and the enzyme is therefore a markedly more efficient catalyst of *p*-NPA hydrolysis than is complex **1**. To some extent, this is accounted for by the difference in the values of K_m for the two systems,

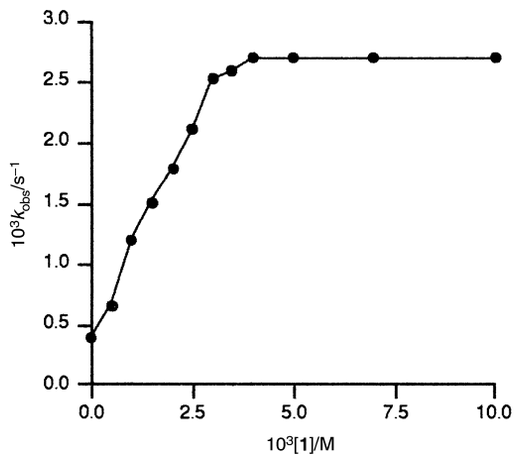


Fig. 5 Plot of k_{obs} for the liberation of *p*-nitrophenolate from *p*-NPA as a function of [**1**]. ([*p*-NPA] = 20 μM, pH 8.20, 55 ± 0.1 °C, aqueous, 50 mM MOPS buffer, I = 0.1 M.)

although, after correcting for the difference in the temperature at which the two values were recorded, CA remains the more efficient catalyst by almost two orders of magnitude. The reason for this is not clear, and further studies (both with enzyme models and with site-directed mutagenesis) may afford an explanation. Taking into account the difference in temperature at which the data were obtained, the value of k_2 for complex **1** is comparable to that for **3** ($k_2 = 4.1 \times 10^{-2}$ M^{-1} s^{-1} at pH 8.2, 25 °C).[9]

Concluding remarks

These results provide an indication of the importance of active-site functionalities to the catalytic activity of CA. They also illustrate the advantage, in terms of catalytic efficiency, which evolution has conferred on the enzyme. In order for a biomimetic catalyst to achieve an efficiency comparable to that of CA, the next generation of such catalysts will require additional functionality. The systematic inclusion of moieties which model other aspects of the enzyme architecture could help to further explain why simple Zn^{2+} complexes are less efficient catalysts than CA, thus bringing the prospect of outstanding 'artificial' enzymes a step closer.

Experimental

^{1}H NMR spectra were recorded using a Bruker AM360 spectrometer operating at 360 MHz. UV–visible spectrophotometric data for kinetic experiments were obtained using either an Hewlett-Packard HP8453 or HP8452 diode-array spectrophotometer, which was operated under PC control with Hewlett-Packard software. Potentiometric titration and pH-stat kinetic data were obtained using a Metrohm 736 GP Titrino apparatus, which was operated under PC control with Metrohm software. *p*-NPA was obtained from Lancaster Synthesis and recrystallised from hexane. The recrystallised material was stored under argon, and used to prepare 50 mM stock solutions in dry acetonitrile, which were freshly-prepared every two days. Good's buffers were purchased from Sigma or Lancaster Synthesis and were used as obtained. Zinc perchlorate hexahydrate was purchased from Aldrich and used as an approximately 2 M aqueous solution, the precise concentration of which was determined by EDTA titration. Tris(aminomethyl)ethane was prepared according to literature procedures.[21] Freshly-distilled water was used throughout. Ionic strength (I) was maintained with $NaClO_4$.

CAUTION: although no problems were encountered during the use of zinc perchlorate or perchloric acid, suitable care and precautions should be taken when handling such potentially explosive compounds.

Kinetic experiments

The liberation of the *p*-nitrophenolate anion was observed spectrophotometrically at 405 nm. A typical kinetic experiment was conducted as follows. A 1 cm quartz cuvette containing 2.5 ml of a buffered solution of the complex was temperature-equilibrated in the spectrophotometer and then treated with an aliquot of a solution of *p*-NPA in dry acetonitrile. The kinetic run was then started and followed almost to completion (>6 half lives) for the determination of the observed pseudo first-order rate constants, k_{obs}, or to the consumption of 1–2% of the ester for the determination of the initial rate, v. The data presented are the mean values of triplicate runs.

Potentiometric titrations

Protonation constants for tris(aminomethyl)ethane (with or without one equivalent (1 mM) of $Zn(ClO_4)_2$) were obtained by titrating an aqueous solution (50 ml, 25.0 °C, I = 0.1 M) of tris(aminomethyl)ethane (1 mM) and perchloric acid (3 mM) under nitrogen with a standard aqueous solution of sodium hydroxide (0.1 M). Each curve shown is the mean of three independent titrations.

Preparation of complex 1 for ^{1}H NMR analysis

A solution of complex **1** (100 ml) was prepared by dissolving tris(aminomethyl)ethane (11.7 mg, 0.1 mmol) in water (95 ml), and the pH was adjusted to *ca.* 6 by the addition of perchloric acid (2 M). The solution was treated, with stirring, with one equivalent of $Zn(ClO_4)_2$ (50 μl of a 2 M solution), and the pH adjusted to 8.80 by the addition of sodium hydroxide solution (6 M). The volume of the solution was made up to 100 ml and a check made to ensure that the pH of the solution was still *ca.* 8.80. An aliquot of the solution (1 ml) was withdrawn, evaporated to dryness, the residue treated with a little D_2O and the whole evaporated to dryness once more. After a second D_2O exchange, the residue was dissolved in 20% v/v D_2O in CD_3CN (1 ml) and the ^{1}H NMR spectrum recorded. (This procedure permits the accurate control of the pH of the solution and avoids the need for the conversion of the pH meter reading to the corresponding pD value.)

Acknowledgements

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References

1 (*a*) *Carbonic Anhydrase*, eds. F. Botrè, G. Gros and B. T. Storey, VCH, Weinheim, 1991; (*b*) J. N. Earnhardt and D. N. Silverman, in *Comprehensive Biological Catalysis*, ed. M. Sinnott, Academic Press, London, 1998, vol. 1, ch. 13i.
2 (*a*) W. N. Lipscomb and N. Sträter, *Chem. Rev.*, 1996, **96**, 2375; (*b*) D. S. Auld, *Struct. Bonding*, 1997, **89**, 29; (*c*) I. Bertini and C. Luchinat, in *Bioinorganic Chemistry*, eds. I. Bertini, H. B. Gray, S. J. Lippard and J. S. Valentine, University Science Books, Mill Valley (California), 1994, ch. 2.
3 R. D. Hancock and A. E. Martell, *Adv. Inorg. Chem.*, 1995, **42**, 89.
4 S. F. Lincoln and A. E. Merbach, *Adv. Inorg. Chem.*, 1995, **42**, 1.
5 Y. Pocker and S. Sarkanen, *Adv. Enzymol.*, 1978, **47**, 149.
6 (*a*) B. Greener, M. H. Moore and P. H. Walton, *Chem. Commun.*, 1996, 27; (*b*) C. Kimblin, W. E. Allen and G. Parkin, *J. Chem. Soc., Chem. Commun.*, 1995, 1813; (*c*) R. Alsfasser, S. Trofimenko, A. Looney, G. Parkin and H. Vahrenkamp, *Inorg. Chem.*, 1991, **30**, 4098; (*d*) A. Looney, G. Parkin, R. Alsfasser, M. Ruf and H. Vahrenkamp, *Angew. Chem., Int. Ed. Engl.*, 1992, **31**, 92.
7 M. Ruf and H. Vahrenkamp, *Chem. Ber.*, 1996, **129**, 1025. For an interpretation of this and related results, see M. Rombach, C. Maurer, K. Weis, E. Keller and H. Vahrenkamp, *Chem. Eur. J.*, 1999, **5**, 1013.
8 Only a few carbonic anhydrase models with catalytic activity have been reported; examples with demonstrated esterase activity include (*a*) E. Kimura, H. Hashimoto and T. Koike, *J. Am. Chem. Soc.*,

1996, **118**, 10963; (*b*) J. Suh, O. Han and B. Chang, *J. Am. Chem. Soc.*, 1986, **108**, 1839; (*c*) J. Chin and X. Zuo, *J. Am. Chem. Soc.*, 1984, **106**, 3687; (*d*) D. S. Sigman and C. T. Jorgensen, *J. Am. Chem. Soc.*, 1972, **94**, 1724.
9 E. Kimura, T. Shiota, T. Koike, M. Shiro and M. Kodama, *J. Am. Chem. Soc.*, 1990, **112**, 5805.
10 (*a*) C. Bazzicalupi, A. Bencini, A. Bencini, A. Bianchi, F. Corana, V. Fusi, C. Giorgi, P. Paoli, P. Paoletti, B. Valtancoli and C. Zanchini, *Inorg. Chem.*, 1996, **35**, 5540; (*b*) C. Bazzicalupi, A. Bencini, A. Bianchi, V. Fusi, C. Giorgi, P. Paoletti, B. Valtancoli and D. Zanchi, *Inorg. Chem.*, 1997, **36**, 2784.
11 For reviews of small-molecule models of a number of different enzymes, see (*a*) E. Kimura and E. Kikuta, *J. Biol. Inorg. Chem.*, 2000, **5**, 139; (*b*) J. K. Bashkin, *Curr. Opin. Chem. Biol.*, 1999, **3**, 752; (*c*) E. Kimura, T. Koike and M. Shionoya, *Struct. Bonding*, 1997, **89**, 1; (*d*) R. Breslow, *Acc. Chem. Res.*, 1995, **28**, 146; (*e*) E. Kimura, *Prog. Inorg. Chem.*, 1994, **41**, 443; (*f*) K. D. Karlin, *Science*, 1993, **261**, 701; (*g*) J. Suh, *Acc. Chem. Res.*, 1992, **25**, 273; (*h*) J. Chin, *Acc. Chem. Res.*, 1991, **24**, 145.
12 A. Cornish-Bowden, *Fundamentals of Enzyme Kinetics*, Portland, London, 1995.
13 (*a*) N. Kitajiri, T. Arishima and S. Takamoto, *Nippon Kagaku Zasshi*, 1970, **91**, 240 (*Chem. Abstr.*, 1970, **73**, 29530); (*b*) G. Anderegg, *Helv. Chim. Acta*, 1962, 1303.
14 H. Sigel and R. B. Martin, *Chem. Soc. Rev.*, 1994, **23**, 83.
15 A quantitative analysis of this observation using thermodynamic data has been reported: T. Itoh, Y. Fujii, T. Tada, Y. Yoshikawa and H. Hisada, *Bull. Chem. Soc. Jpn.*, 1996, **69**, 1265.
16 The nature of the donor atoms is also important; thus a complex composed of a tripodal array of sulfur donors does not appear to afford a pK_a reduction: C. Kimblin, B. M. Bridgewater, D. G. Churchill and G. Parkin, *Chem. Commun.*, 1999, 2301.
17 Y. Pocker and J. T. Stone, *J. Am. Chem. Soc.*, 1965, **87**, 5497.
18 C. A. Fierke, J. F. Krebs and R. A. Venters, in *Carbonic Anhydrase*, eds. F. Botrè, G. Gros and B. T. Storey, VCH, Weinheim, 1991, pp. 22–35.
19 X. Xu, A. R. Lajmi and J. W. Canary, *Chem. Commun.*, 1998, 2701.
20 B. Zhang and R. Breslow, *J. Am. Chem. Soc.*, 1997, **119**, 1676. (See also: B. Zhang and R. Breslow, *J. Am. Chem. Soc.*, 1998, **120**, 5854).
21 (*a*) R. J. Geue and G. H. Searle, *Aust. J. Chem.*, 1983, **36**, 927; (*b*) E. B. Fleischer, A. E. Gebala, A. Levey and P. A. Tasker, *J. Org. Chem.*, 1971, **36**, 3042.