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Biological EXAFS at room temperature

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Biological EXAFS experiments are usually carried out on frozen samples in order to minimize radiation damage. This causes difficulties when studies at different pH are made, since the pH cannot be measured directly for the frozen sample.

We have carried out EXAFS studies of Human Carbonic Anhydrase, a zinc metalloenzyme, at room temperature using a rastering technique to move the X-ray beam across the sample and so minimize radiation damage. The catalytic activity of this enzyme is strongly dependent on the pH and so samples in solutions with different pH values were investigated to determine any structural changes which may occur. The analysis used restrained refinement with full inclusion of multiple scattering.

The structural changes observed with varying pH are very small.

Keywords: metalloenzymes, structure

1. Introduction.

The structure of metalloenzymes has been a major topic of investigation for 40 years or more: the diversity of catalytic functions exhibited by such enzymes makes them of major biological interest. In many cases the metal ion is present in the active site and the site specificity of EXAFS is then a major advantage in understanding the behaviour of the metal environment during the catalytic process. Since EXAFS studies may be made in solution, structural information on the enzyme in different states, for example at different pH values (the pH radically alters the catalytic activity in many cases) may be obtained.

The carbonic anhydrases (EC 4.2.1.1) are a family of zinc metalloenzymes, ubiquitous in nature, which catalyze the hydration of carbon dioxide. The enzymes studied here are monomers of about 29 kDa each of which contains one catalytically-essential zinc atom at the active site. The catalytic reaction rate is strongly dependent on the pH of the solution: indeed the direction of the reaction may easily be reversed by changing the pH.

Heretofore, one limitation on the use of EXAFS has been the use of frozen samples to minimize radiation damage. This makes determining the pH of the sample difficult if not impossible. Here we report the results of an EXAFS study undertaken at room temperature on samples of type I Human Carbonic Anhydrase (HCAI) at several known pH values.

2. Experimental Method.

The native form of HCAI was prepared by affinity chromatography (Chegwidden, 1991) and concentrated to about 2mmol (60mg/ml) in aqueous solution. Samples were prepared at different pH values by mixing with the appropriate quantity of 5 mmol Tris-HCl and reconcentrating. For EXAFS measurements the samples were injected into standard perspex sample holders fitted with mylar windows. These give a solution thickness of about 1mm with a window 1cm x 0.5 cm. All experiments were carried out at room temperature.

EXAFS data from the zinc K edge were obtained on station 8.1 of the Daresbury SRS. Beam currents during data taking were about 200mA. Station 8.1 has a double crystal silicon (220) monochromator and provides a focussed beam of about 1mm diameter at the sample. The secondary fluorescence photons were detected using a 13 element Canberra solid state detector with the energy window set to the zinc K_α emission. This configuration resulted in a signal to background ratio of about 10 and a signal count of 5000 photons/sec. Data were taken out to a photoelectron k of 15 A^{-1}. Counting times varied between 2 and 15 secs per point, increasing proportionally with k. To obtain good quality data eight spectra, each taking about 30 min, were averaged for each sample.

In order to minimize possible radiation damage, the sample holders were mounted on a translation stage so that they could be moved relative to the beam in both the vertical and horizontal directions. This movement was done by hand in this study, although the system could easily be motorized. The samples were moved either after every scan, or after every two, four or eight scans and the results checked against one another to search for signs of radiation damage.

The EXAFS data were calibrated and background-subtracted using standard Daresbury software and analyzed using the Daresbury program EXCURV92. Fast curved wave theory (Gurman, et al 1984) was used throughout, with scattering parameters calculated within the program from a complex, energy-dependent Hedin-Lundqvist potential. Multiple scattering (Gurman, et al 1986) was included within the imidazole rings. Restrained refinement (Binsted, et al 1992), where the shape of the ring is restrained to a form close to the ideal by the imposition of a fitting penalty, was used to limit the number of free parameters. With this form of analysis the variables are essentially two ring orientation parameters and the length of the Zn-N bond.

3. EXAFS Results.

The results of the EXAFS analysis of samples showing no structural changes during data taking (see discussion below) are summarized in Table 1. The 100K data is taken from Amiss, et al (1997). Figure I shows an example of the quality of the data and of the fits obtained. The fits to the first FT peak of all spectra analyzed were consistent with a zinc cordination of three nitrogen and one oxygen atoms when the amplitude reduction factor AFAC was set equal to unity. Hence the Hedin-Lundqvist potential gives a good description of the loss processes, no further correction being necessary. The ring orientation parameters θ and ϕ, together with the atom indexing, are defined in Figure 2. Since we cannot distinguish between N and C scattering atoms, the sign of ϕ cannot be determined by EXAFS.

4. Discussion.

The major purpose of this study was to investigate the possibility of doing biological EXAFS at room temperature, with radiation damage minimized by moving the beam across the sample between scans. No structural changes were observed when the samples were moved after every scan (30 mins in beam) or every other scan. Slight changes were observed when the samples were moved after every fourth scan, particularly in the Debye-Waller factors of the nearest neighbours. Thus, for HCAI, we suggest that significant radiation damage occurs in samples exposed to the monochromatic beam for between one and two hours. This time will, of course, vary between materials: our technique allows checks to be made for such damage and for corrective measures (shorter exposures) to be applied.

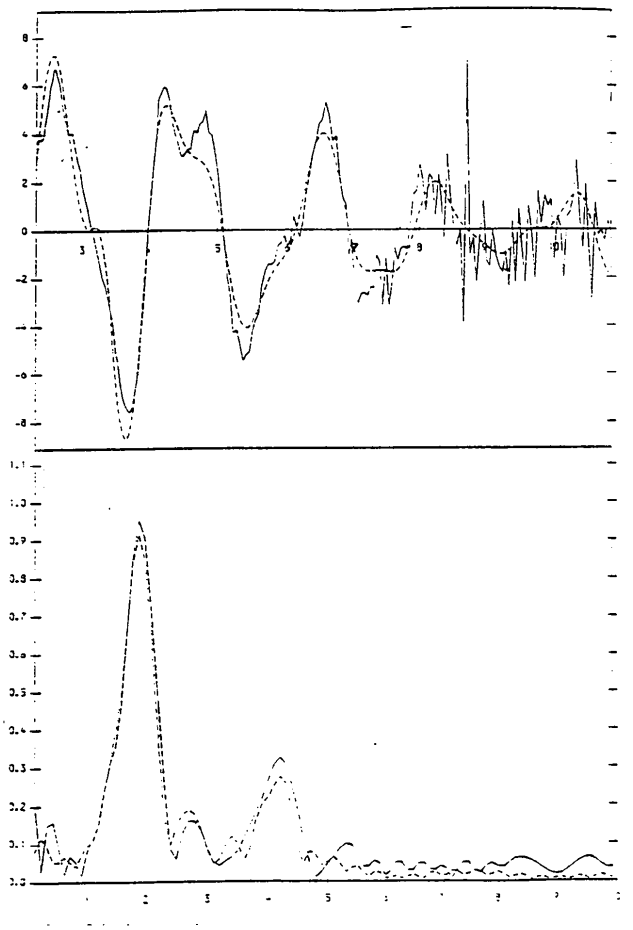

Figure 1 Zinc K edge EXAFS $k^3\chi(k)$: experiment (solid line) and fit (dashed line). HCAI at room temperature and pH 9.

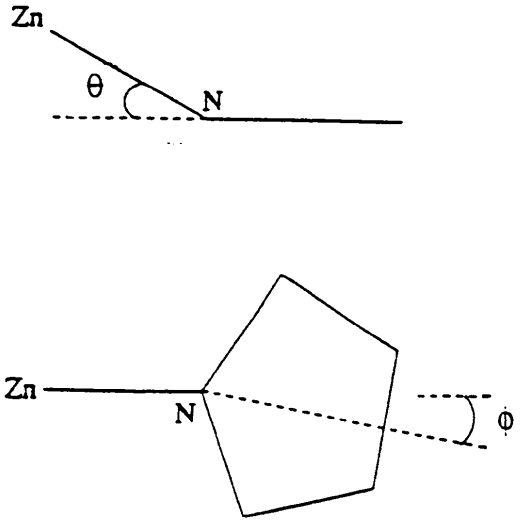

Figure 2 Definition of ring orientation parameters.

Table 1. Radial distances, and ring orientation angles, in HCAI

	100K pH 7	RT pH 5	RT pH 7	RT pH 9	
O	1.82	1.80	1.84	1.82	R±0.02 A
	40	50	60	70	$(\sigma^2\pm30)10^{-4}A^2$
N_1	2.02	2.01	2.00	2.00	R±0.02 A
	20	25	35	50	$(\sigma^2\pm20)10^{-4}A^2$
C_2	2.89	2.87	2.87	2.91	R±0.05 A
C_3	3.16	3.12	3.14	3.15	R±0.05 A
N,C_4	4.14	3.99	4.02	4.07	R±0.05 A
N,C_5	4.24	4.22	4.22	4.21	R±0.05 A
ϕ	15	18	17	13	$\pm5^o$
θ	38	47	44	46	$\pm5^o$

Our results show only very small changes in the local environment of the zinc atom as the pH is varied from acid to alkaline, even though the direction of the hydration reaction which the enzyme catalyzes is reversed by such a change. There are no significant changes in the Zn-N and Zn-O distances. There is a small, and just significant, change in the Debye-Waller factors for these coordinations, these showing an increase with pH: this may indicate a steady weakening of the bonds. There are no significant changes in the ring orientations.

In our previous study of different forms (HCAI, II and III, with very different catalytic activities) of HCA (Amiss, et al 1997) we found no difference between the several zinc environments. We have here extended this study to investigate the effect of acidity and have again found essentially no difference in the zinc environment in HCAI in acidic, neutral and alkaline solutions despite the strong effect of pH on catalytic activity.

5. References.

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