Specific Inactivation of Solid Carbonic Anhydrase upon X-Ray Resonance Absorption in the Constituent Zinc Atom[1a]

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Abstract: The inactivation of bovine carbonic anhydrase irradiated in the solid phase with monoenergetic low-energy x-rays has been investigated. Upon irradiation of the metalloenzyme, efficient inhibition of its esterase activity was observed. The inactivation normalized to an equal dose absorbed was shown to depend on the x-ray energy. For photon energies slightly above the Zn K edge, the inactivation significantly exceeds that expected on the basis of energy absorption. This amplifier effect is interpreted in terms of Auger charging resulting from inner-shell vacancies in zinc atoms. The concomitant loss of zinc atoms from the enzyme molecules upon irradiation was also studied by means of ion-exchange chromatography and parallels the photon-energy dependence of enzyme inactivation. Complete and spontaneous recovery of enzyme activity occurs within an hour after dissolution which may be interpreted as a slow conformational change of the native enzyme subsequent to dissolution.

Nuclear chemists have long been acquainted with the drastic damaging consequences of inner-shell ionization (for a review cf. ref 2). If a radionuclide undergoing a nuclear process associated with inner-shell ionization, e.g., K capture, belongs to an atom bound in a molecule, the latter has a small chance to survive. The problem is also of significant importance for radiobiology and nuclear medicine,[3] in view of the increasing utilization of isotopes decaying by K capture, in particular ^{123}I and ^{125}I. Biophysical implications are greatly increased in importance by the fact that selected inner-shell vacancies can also be formed when monoenergetic low-energy x rays interact with matter via the photoelectric effect. Electronic readjustment that inevitably follows is essentially the same, whatever the mode of a primary inner-shell ionization. The result is an Auger vacancy cascade and multiple charging of the affected atom.[4] While in the few molecular systems studied in the gas phase the molecule eventually explodes because of Coulomb repulsion,[5] little is known about the primary chemical consequences of Auger charging in condensed phases.[6-8] The biological and biochemical effects of such multiple charging in biomolecules are of particular interest because of the obvious implications for radiation therapy of metabolic dysfunctions.[7,9] A resonance response of biomolecules to the absorption of low-energy x rays has been discovered.[7,9,10] This radiation resonance effect can be summarized as follows. The same dose absorbed produces different radiation damage as the energy of incident photons changes, the maximum effect being observed at a photon energy just above the K-absorption edge of the absorbing constituent atom. There is considerable evidence that the specific consequences of the Auger charging are responsible for this "amplifier effect".

Biomolecules composed almost entirely of light atoms and one heavy atom are particularly suitable objects for this type of experiment. An especially important class of biologically active molecules that contain heavy atoms is that of the metalloenzymes. In the present study, we have investigated the effect upon the enzymatic activity of solid bovine carbonic anhydrase (BCA) of the absorption of monoenergetic x rays of variable energy and correlated it with a fraction of zinc released from BCA.

Adequate studies necessitate accurate dosimetry and the use of x radiation from an adjustable monoenergetic source. Less meaningful is the method applied by Addink,[11] who irradiated a solution of carbonic anhydrase in water with a continuum of x rays, regulating only the maximum wavelength by changing the tube voltage. Addink has shown that polychromatic x rays, whose maximum energy lies at 9 keV, are significantly less effective in releasing zinc from carbonic anhydrase than x rays with $E_{max} = 10.5$ keV at equal irradiation time. However, he did not work with monochromatic radiation, and no dosimetry data or absorption calculations were presented.

Experimental Section

Crystalline bovine carbonic anhydrase (mol wt 2.9×10^4) was obtained from Boehringer Mannheim GmbH and used without further purification.

For irradiations, monoenergetic x-ray beams of different energies from fluorescent targets were used. The irradiation arrangement, as well as the dosimetric measurements, was described previously.[7] X rays of energies just below the K edge of zinc were fluorescent x rays from Cu and Zn targets (8.14 and 8.74 keV, respectively) and, just above the K edge of zinc, from Ge and As targets (10.01 and 10.69 keV).

Esterase activity determinations were carried out by dissolving 3-5 mg of the enzyme in 100 ml of 0.025 M Tris buffer (pH 7.5). *p*-Nitrophenyl acetate (0.1 ml) solution (10^{-2} M, in acetonitrile) was added to 3 ml of enzyme solution in the cuvette of a Cary 14 recording spectrophotometer, and the esterase activity was calculated from the first-order rate constant of the increase in absorbance at 400 nm. In the blank runs, 0.1 ml of substrate was added to 3 ml of Tris buffer without dissolved enzyme.

For zinc release measurements, 60-70 mg of irradiated BCA, dissolved in tridistilled water, was introduced onto a Chelex 100 (Bio-Rad Laboratories) column, preconditioned with a buffer solution CH_3COOH + NaOH (pH 6.5), and a buffer solution was used as the first eluent. The enzyme was eluted from the column with the effluent flow, while unbonded zinc was retained. The latter was then removed with 0.1 M HCl and extracted to dithizone/CCl_4 using a 7% solution of bis(2-hydroxyethyl)dithiocarbamate as a masking agent. The determination was carried out spectrophotometrically at 532 nm (Beckman's ACTA III uv spectrophotometer). With this method, a few micrograms of Zn can be determined with an accuracy of ±1%.

It should be noted that, in contrast to Addink,[11] we found in carbonic anhydrase no loosely bound zinc that could be separated by shaking with dithizone at pH 6.5.

Results

After irradiation with monochromatic x rays in the solid phase, the enzyme was dissolved in Tris buffer and its esterase activity determined photometrically. Initially, we expe-

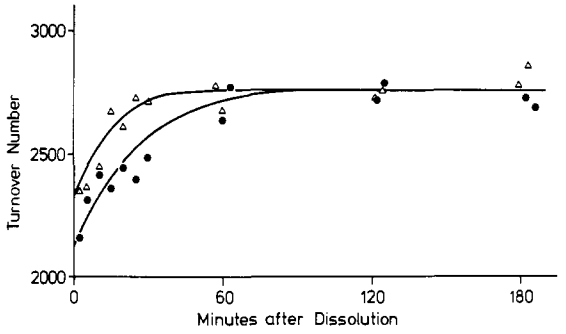

Figure 1. Recovery of enzyme activity: (△) unirradiated; (●) irradiated with As-fluorescent x rays, 4.38 × 10^4 rads/μmol.

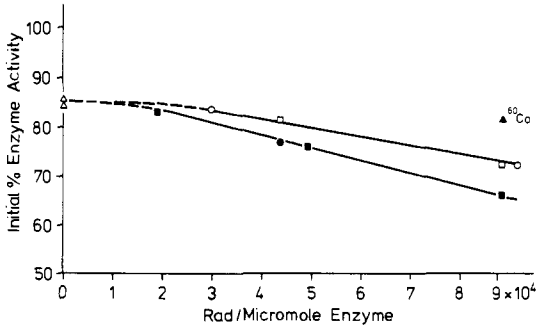

Figure 2. Inactivation of carbonic anhydrase: (□) Cu fluorescence (8.14 keV); (○) Zn fluorescence (8.74 keV); (■) Ge fluorescence (10.01 keV); (●) As fluorescence (10.69 keV); (△) unirradiated.

rienced great difficulty in obtaining reproducible turnover numbers even for the unirradiated enzyme. We finally discovered that freshly dissolved enzyme only slowly attains its full activity. In both irradiated and unirradiated samples, approximately 1 h was required to reach full activity. In the irradiated samples, the initial activity was lower than in the controls. We therefore conducted a series of activity determinations at various times after dissolution for each sample and utilized for subsequent calculations the extrapolated turnover number at the instant of dissolution (Figure 1).

In Figure 2, the initial activity is shown as a function of radiation dose at various photon energies. It is evident that there is a linear relationship between absorbed dose and enzyme inactivation over the dose range studied (from 2 × 10^4 rad/μmol on). Moreover, at equal absorbed dose, radiation whose energy is just *above* the K edge of zinc (9.66 keV), and therefore is absorbed predominantly via a photoeffect in the K shell of zinc, is significantly more effective in inactivation of the enzyme than radiation of slightly lower energy (which is preferentially absorbed in the L shell of zinc). An even smaller effect per unit absorbed dose is observed for ^{60}Co γ rays with an average energy of 1.25 MeV.

We also found that zinc is released from BCA as the consequence of irradiation with low-energy x rays. The energy dependence of this effect qualitatively parallels those of enzyme inactivation (Figure 3). Effect vs. energy curves for both inactivation and zinc release show a sudden jump at the energy corresponding to the binding energy of K-shell electron in zinc atom.

Discussion

We consider it probable that the slow development of enzyme activity after dissolution (Figure 1) is indicative of a slow conformational change. A similar phenomenon has recently been observed for phosphoribosyl-ATP synthetase.[12] The suggestion that the conformation of solid carbonic anhydrase differs from its conformation in solution might be of interest in the context of present efforts to correlate crystal structure and binding of substrate at the active site of human carbonic anhydrase.[13] An alternative explanation for the phenomenon of slow activity development might be that the enzyme dissolves as a dimer or some sort of oligomer which dissociates slowly. It is also conceivable that in the crystal, the active site does not have the full zinc content and that the increase in activity is due to zinc binding upon dissolution.

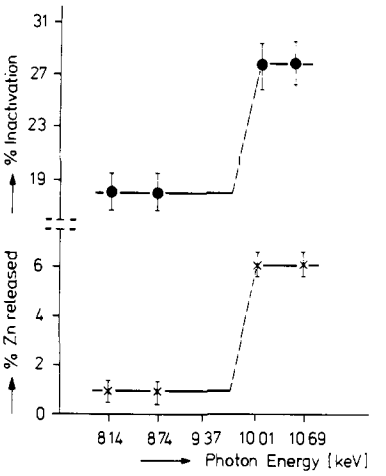

Figure 3. Percentages of inactivation and of zinc. Release vs. photon energy, normalized to an absorbed dose of 3 × 10^4 rads/μmol. The error bars represent the standard deviation of the mean of four determinations.

A plot of the enzyme activity after irradiation vs. dose (Figure 2) shows that dose-response curves are linear, except for a small shoulder at very low doses. Moreover, there is a distinct difference in the yield of inactivation by photon energies below and above the K edge of zinc. When the results are normalized to equal dose absorbed and plotted vs. x-ray energy, the resonance effect in inactivation become apparent. This relationship is seen best in Figure 3. It is known that, in the energy range of interest, the cross section for absorption of x rays varies with energy in such a way that absorption edges appear. Consequently, the calculated mass absorption coefficient of the BCA molecule abruptly increases by 14% at 9.66 keV because of the K edge of the constituent zinc atom. If this is taken into account by plotting inactivation vs. photon energy at equal absorbed dose, then a straight line would be expected in the absence of damage amplification. In actuality, upon crossing the K edge, the inactivation per 3 × 10^4 rad/μmol absorbed increases from 18 to 28%, thus demonstrating a specific additional "Auger inactivation".

We also found that the molecular changes associated with inactivation involve zinc release from BCA. Again, upon crossing the K edge, the fraction of zinc released from BCA increases from 0.9 to 6.1%. While the zinc atom in BCA represents only 0.2% of the molecule's weight, it absorbs 2% of the total x-ray energy below the zinc K edge, and 15% just above the K edge. The amount of enzyme inactivation which is caused by the fraction of radiation absorbed in zinc, relative to the damage done by the 85% of absorbed dose that is deposited in the rest of the molecule at energies slightly above the K edge, can be estimated from a plot of inactivation vs. zinc release, both per the same 3 × 10^4 rad/μmol absorbed. At extrapolated zero zinc release, which we assume to correspond to zero absorption of the radiation in zinc, 16% inactivation remains (as compared with 28% at the maximum absorption in zinc). The inactivation

due to that 15% of total dose which is absorbed in zinc slightly above the K edge, therefore, causes 43% (i.e., (28 − 16)/28 × 100%) of the total damage. It is apparent that x-ray resonance absorption in the metal atom is approximately three times as effective in inactivating the enzyme as absorption within the rest of the molecule.

This amplifier effect, in analogy to that observed earlier for iodine yield from iodoamino acids[9] and free radical yield in 5-bromodeoxyuridine,[7] is indicative of the drastic consequences of Auger charging following the photoeffect specifically induced in a heavy atom of a biomolecule. In this context it should be noted that ^{60}Co γ rays, which are almost entirely absorbed via the Compton process, exhibit only one-half the inactivation efficiency of 9 keV x rays, and one-third that of 10 keV photons at equal absorbed doses (Figure 2). Therefore, the absorption in the keV (photoelectric) energy region is significantly more effective in inactivating the enzyme than absorption of high energy radiation.

We assume that the primary chemical consequence of x-ray resonance absorption in the K shell of zinc is the ejection of the Zn atom from the active site where it is liganded to three histidyl residues.[13] Since recovery is spontaneous and complete, no significant damage appears to ensue to the primary structure of the enzyme as a result of the high positive charge on the zinc atom. One reason for this stability might be the fact that the histidine ligands, being Lewis bases, should be able to facilely donate electrons for partial charge neutralization on the zinc. Assuming that the maximum charge which can be imparted to the zinc atom as a consequence of the vacancy cascade originated in the K shell is +5, the transfer of one electron from each of the histidyl residues would leave a doubly charged zinc ion that is no longer bound to, and in fact repelled by, the histidyl moieties. On the other hand, when photons of energy below the K edge of zinc are absorbed, this can only lead to a lower Auger charge. In this case, zinc release is not very probable and indeed we observe only about 15% of the zinc which is released upon absorption of the same dose above the K edge.

What are the implications of our observations for further studies of the biochemical and biological consequences of x-ray resonance absorption? The technique is unique in permitting the deposition of well-defined amounts of energy in specific and identifiable positions of a molecule. Such precisely localized energy deposition might be utilized for "molecular surgery" in which selected portions of the molecule are altered or destroyed. In metalloenzymes, including carbonic anhydrase, the amount of energy can be varied by substituting other metal atoms.[14,15] Even more interesting is the possibility of introducing heavy atoms into various positions in enzyme substrate molecules and probing the active site of the enzyme by studying the effects upon subsequent enzyme activity of the absorption of energy in different positions of the bound substrate molecule.

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References and Notes

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