MACROMOLECULAR CONFORMATION IN SOLUTION

STUDY OF CARBONIC ANHYDRASE

BY THE POSITRON ANNIHILATION TECHNIQUE

EDWARD D. HANDEL AND GEORGE GRAF, *Department of Biochemistry*
JAMES C. GLASS, *Department of Physics, North Dakota State University, Fargo, North Dakota 58105 U.S.A.*

ABSTRACT The structural features of carbonic anhydrase (carbonate hydro-lyase; EC 4.2.1.1) in aqueous solutions were probed by the positron annihilation technique. The data obtained under varying conditions of temperature, pH, and enzyme concentration were interpreted in terms of the free volume model. The change of enzymic activity with temperature is accompanied by a change in free volume of the protein. Upon thermal denaturation an irreversible change in free volume of the molecule occurred. At low temperatures the protein-water interactions were investigated. These results are discussed in terms of current concepts of structure-function relationships in proteins. This study shows the sensitivity of the positron annihilation method toward the structure of proteins related to their overall conformation and to the nature of bound water.

INTRODUCTION

Positron annihilation has developed into an extremely useful probe of chemical systems. The positron, as the antiparticle of the electron, can annihilate with an electron, their combined mass being completely converted into energy in the form of γ-rays. The annihilation process and its particular characteristics reflect the properties of the annihilating electron and of the local environment in which the annihilation occurs. Several surveys of positron annihilation theory, methodology, and its application are available (Ache, 1972; Merrigan et al., 1972; Stewart and Roellig, 1967; Green and Lee, 1964; Graf et al., 1979).

The successful application of the positron annihilation method in the study of chemical systems is due in part to the development of the free volume model associated with the pick-off annihilation quenching mechanism (Brandt, 1967; Goldanskii, 1967). This technique is sensitive to changes in free volume caused by pressure or by thermal expansion in the same phase and often shows significant changes during phase transitions. For our purposes here, the measurable parameters of importance are I_2 and τ_2. I_2 is the relative probability that the positron will enter a material, form ortho-positronium (o-Ps) in a free volume region, or region of relatively low electron density, pick off another electron through an attractive spin-spin

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Dr. Handel's present address is the Department of Chemistry, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061.

Abbreviations used in this paper: BCA-B, bovine carbonic anhydrase isozyme B; keV, thousand electron volt; MCA, multichannel analyzer; MeV, million electron volt; o-Ps, ortho-positronium; p-Ps, para-positronium; Ps, positronium; SCA, single channel analyzer; TPHC, time-to-pulse-height converter.

interaction, and then rapidly annihilate. The associated lifetime τ_2 lies between the extreme limits of 140 ns for ortho-positronium and 0.1 ns for para-positronium (p-Ps).

The application of the positron annihilation technique to biophysical problems is rather recent (cf. Handel et al., 1976; McMahon et al., 1979; Graf et al., 1979). Here we investigate our suggestion that the positron is a sensitive probe of protein conformation. The model protein of this study, carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1), is one of the better characterized enzymes. Its structural, functional, and physiological characteristics have been reviewed in great detail (Lindskog et al., 1971; Coleman, 1973; Pocker and Sarkanen, 1978). In spite of the progress in understanding many of the kinetic features of carbonic anhydrase mechanism, several structural and energetic questions of great importance still remain unanswered. Conformational fluctuations presumably play a major role in linking protein structure to biological function (Handel, 1977). The models proposed in the literature should be tested experimentally by measuring the parameters involved in these transitions. One of these parameters, the conformational volume, can be directly probed by the positron annihilation technique and related to the temperature or pH-induced variations of biological activity.

METHODS

Carbonic anhydrase from bovine erythrocytes (BCA; Sigma Chemical Co., St. Louis, Mo.) was further purified and separated into its isozymes (BCA-A and BCA-B) by preparative polyacrylamide disk gel electrophoresis. The BCA-B fraction was dialyzed against distilled water and lyophilized. This isozyme was stored at −15°C in a desiccator. Activity measurements showed that the enzyme preparation was stable under storage conditions.

Steady state data for the temperature dependence of the BCA-B catalyzed hydrolysis of *p*-nitrophenyl acetate were obtained by the stopped-flow method (Pocker and Stone, 1967).

Enzyme solutions for positron annihilation studies were prepared by dissolving lyophilized BCA-B in deionized distilled water. The final enzyme/water ratio in the concentration dependence studies varied between 10 mg/ml and 1,000 mg/ml. Other studies were performed using an enzyme/water ratio of 500 mg/ml.

Samples for pH dependence studies were prepared by dialyzing the protein solution against a 4-liter volume of deionized distilled water adjusted to the desired pH with dilute sodium hydroxide solution. The pH of the dialyzand was checked periodically and the dialysis continued until the solutions equilibrated and the pH reached a constant value.

The carbonic anhydrase sample solution was carefully degassed to remove all traces of dissolved oxygen. The degassed sample was sealed under nitrogen atmosphere in a glass sample tube having a diameter 10-mm diam and 70-mm length. This sample tube provided a sample thickness of >200 mg/cm^2 necessary to stop positrons emitted from ^{22}Na.

The temperature was controlled to ±0.5°C over the range −15°C–60°C using a circulating temperature bath. The pH-dependence experiments were conducted at room temperature maintained at 20°C.

Sodium-22 was used as the positron source. Carrier-free sodium-22 chloride (New England Nuclear, Boston, Mass.) was sealed in a thin-walled (<0.1 mm wall thickness) glass capillary tube and positioned in the center of the sample solution. The activity of the source employed in the positron annihilation experiments was 10–15 μCi, giving a ratio of random to real events of 10^{-2}.

The predominant mode of decay of ^{22}Na (halflife, 2.6 yr) produces a positron with an average kinetic energy of 120 keV, a 1.28 MeV γ-ray, and a neutrino. A 1.28-MeV γ-ray is emitted within 10^{-11} s of the positron emission and is experimentally considered to occur simultaneously, providing a convenient signal for the birth of a positron. The annihilation of the positron is signaled by the 0.51-MeV annihilation γ-rays, its lifetime being the time elapsed between these two events.

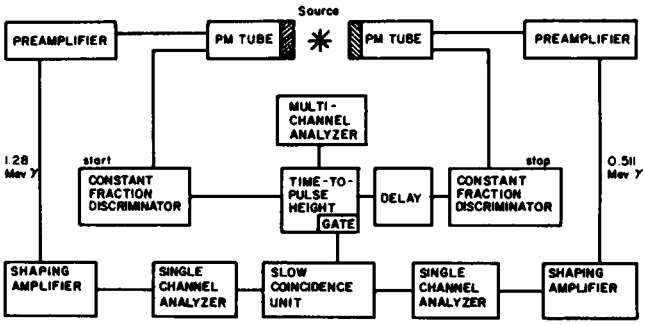

FIGURE 1 Schematic of the fast-slow coincidence system. Timing is performed by the fast inner circuit and energy discrimination by the slow outer circuit.

Positron lifetimes were measured using a fast-slow coincidence spectrometer. A block diagram of this system is shown in Fig. 1. To achieve optimal timing characteristics, the energy discrimination and timing measurement were performed by two separate circuits. Detection of γ-rays using a NATON 136 plastic scintillator of 3.8 cm diam and 1.7 cm thickness (Nuclear Enterprises, Inc., San Carlos, Calif.) results in fast anode signals used in the fast timing circuit.

Dynode signals from the photomultiplier tubes were amplified and routed through the slow circuit to the single channel analyzers (SCA) where energy discrimination was performed. Each SCA was previously adjusted by setting energy windows such that an output signal was generated only if the γ-ray detected had an energy within a certain preset range. The start-side SCA was set to accept signals arising from 128 MeV γ-rays and the stop-side SCA was set to accept signals arising from the detection of an annihilation γ-ray (0.51 MeV). When both energy requirements were met, output signals from both analyzers produced a coincidence in the slow coincidence unit which then triggered the release of the timing pulse temporarily stored in the TPHC. This signal was transformed from analog to digital form and stored in a 256-channel multichannel analyzer (MCA). The collection of 5×10^5 coincidences produced a lifetime spectrum which was resolved into two components:

$$N(t) = N_1 \exp(-\gamma_1 t) + N_2 \exp(-\gamma_2 t) + B,$$

where γ_1 and γ_2 are the annihilation rate constants (reciprocal values) for the two modes of annihilation, N_1 and N_2 are characteristic constants for each component, and B is the background resulting from random coincidences. A short lifetime component (τ_1) corresponding to the rapid free annihilation and p-Ps self annihilation, and a long lifetime component (τ_2) corresponding to the slower o-Ps annihilation were determined using an iterative least squares data reduction scheme (J. B. Cumming, Brookhaven National Laboratory, BNL Report 6470). The program also divides the total area of the annihilation spectrum into two components, I_1 and I_2, after subtraction of random background B, and normalizes so that $I_1 + I_2 = 1$. I_2 thus represents the fraction of annihilation events due to the slower o-Ps annihilation, and I_1 is due to short lifetime processes.

RESULTS AND DISCUSSION

Treatment of the data from positron annihilation lifetime measurements requires the deconvolution of a multiexponential expression. In our study, the best fit to lifetime data was obtained by resolving the spectra into two components by an iterative least squares method. The long-lived component with the lifetime τ_2 results from quenched ortho-positronium. The other component has a much shorter lifetime, τ_1, and is a composite of para-positronium and free annihilation. The lifetime of this component remained constant (0.40 ± 0.05 ns) over the temperature and pH ranges studied and was not further resolved. The timing resolution was

estimated by recording the "prompt" coincidence spectra of ^{60}Co. These spectra were Gaussian, with a full-width-at-half-maximum (FWHM) of 360 ps.

The temperature dependence of the long-lived component as a function of (BCA-B) concentration is shown in Figs. 2, 3, 4, and 5. The lifetime parameters for pure water are shown in each figure for comparison and are in good agreement with other reported values (Fabri et al., 1963; cf. Stewart and Roellig, 1967).

The intensity parameter, I_2, is significantly below the value observed in pure water for all BCA-B concentrations and throughout the entire temperature range. This parameter is related to the number of sites available for Ps formation in the sample. At temperatures above 10°C, there are only slight changes in the number of these sites in the protein-water system. Pure water shows a gentle increase over this temperature range. The lower I_2 value and its insensitivity in this region is interpreted as an ordering effect of the macromolecule on the water.

Above 10°C the temperature dependence of τ_2 reflects two different trends, dependent on

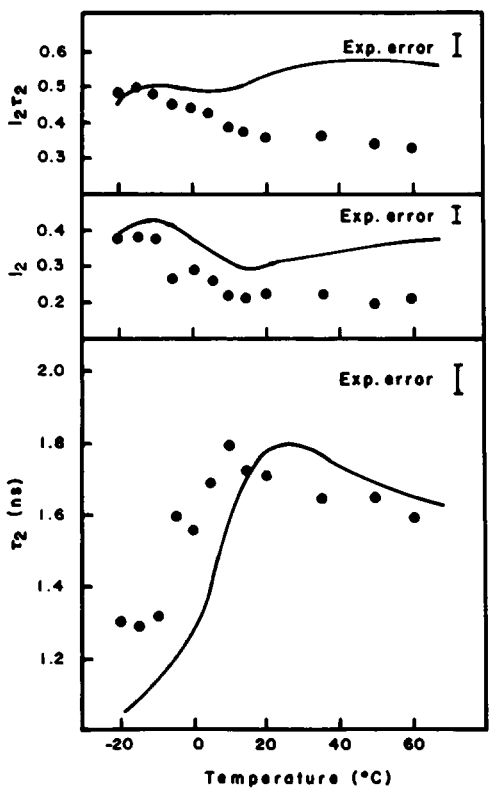

FIGURE 2

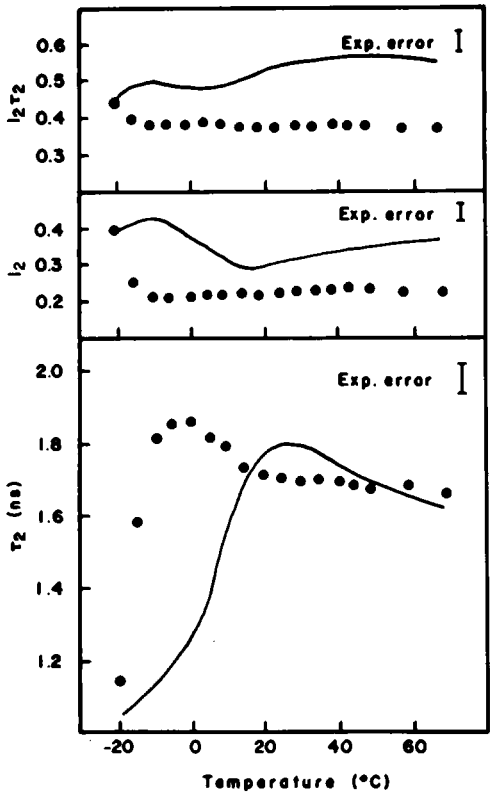

FIGURE 3

FIGURE 2 Temperature dependence of the pick-off annihilation parameters in the 10-mg BCA-B/1,000-mg H_2O system. The solid line indicates parameter behavior for pure water.

FIGURE 3 Temperature dependence of the pick-off annihilation parameters in the 250-mg BCA-B/1,000-mg H_2O system. The average water-to-ice transition is 0°C. The solid line indicates parameter behavior for pure water.

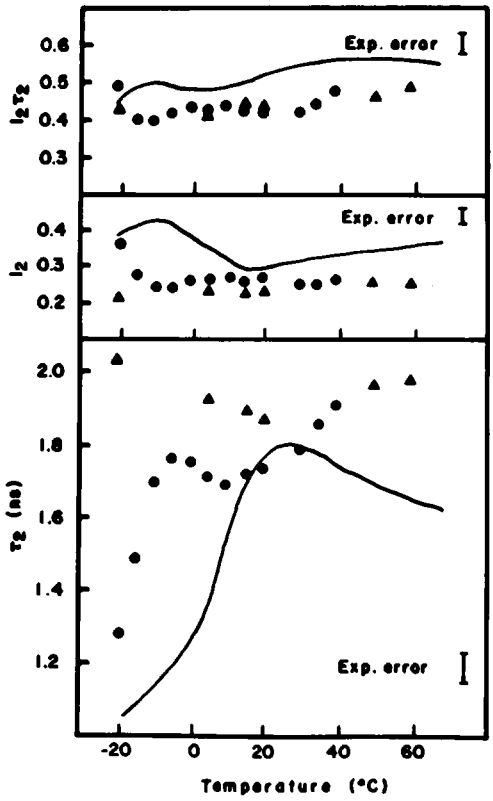

FIGURE 4

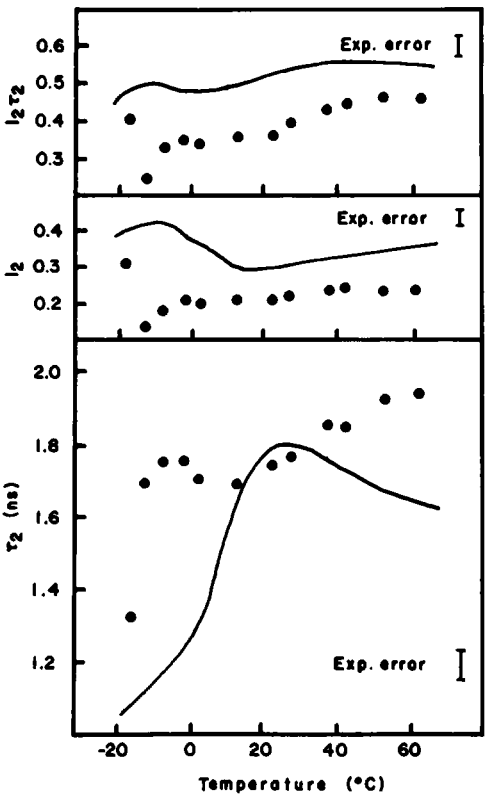

FIGURE 5

FIGURE 4 Temperature dependence of the pick-off annihilation parameters in the 500-mg BCA-B/1,000 mg H_2O system. The Δ data points were obtained by decreasing the temperature after denaturation of the enzyme. The solid line indicates parameter behavior for pure water.

FIGURE 5 Temperature dependence of the pick-off annihilation parameters in the 1,000-mg BCA-B/1,000 mg H_2O system, with increasing temperature. The solid line indicates parameter behavior for pure water.

concentration. The τ_2 lifetimes of the 10 mg/ml (Fig. 2) and 250 mg/ml (Fig. 3) are essentially the same as water above 20°C, reflecting the low protein concentration. The τ_2 values of the 500-mg/ml (Fig. 4) and 1,000-mg/ml (Fig. 5) samples pass through a local minimum at 10°–15°C, increase steeply to 40°C and gradually level off above this temperature, reflecting the contribution due to positron annihilation in the protein. Activity measurements indicate that exposure of the samples to temperatures above 40°C results in irreversible thermal denaturation. Preincubation of the sample at the temperature to be investigated was performed to ensure that all data collected, especially at higher temperatures, were of unchanging samples; all data collected above 40°C were obtained with inactive denatured protein. Some activity loss (~20%) occurred in the 40°C samples but all studies below this temperature retained full activity and showed no hysteresis of the lifetime parameters upon reversing the temperature. Thermally denatured BCA-B, however, showed a large hysteresis effect below 40°C (Fig. 4). Denatured BCA-B has a much larger τ_2 value (~1.9 ns) suggesting the presence of large structural voids or cavities. The $I_2\tau_2$ product also shows an

increase indicating the overall expansion of the molecule. The denatured BCA-B reflected a small temperature dependence suggesting that the denatured protein retains some specific conformational features and may be energetically confined in an inactive distorted conformation.

The trend in the τ_2 dependence on temperatures between 10° and 40°C is similar to the activity-temperature profile of BCA-B (Fig. 6). In both, a gradual increase (over the range 10°–25°C) becomes much more rapid up to 40°C. Activity is rapidly lost at higher temperatures. Thermal denaturation is accompanied by a further increase in τ_2, leveling off at a value of ~2.0 ns. These results indicate that low activity at 10°C may be due to the compactness of the molecule, evidenced by significantly lower values of τ_2 (~1.7 ns at 10°C) compared to the higher activity at 40°C (~1.95 ns). The total conformational volume is a function of the $I_2\tau_2$ product. The 1,000-mg/ml sample (Fig. 5) undergoes a more drastic increase between 10° and 40°C than the 500-mg/ml sample (Fig. 4) although the plateau is at about the same value, $I_2\tau_2 \simeq 0.45$). This is again reflective of effects due to higher protein concentration. The more dilute samples (Figs. 2, 3, and 4) show effects due to bulk water and protein bound water, which may be viewed in terms of the shell water model (Gurney, 1962).

Calculation of a monomolecular A-shell for BCA-B, assuming a spherical shape with a radius of 20 Å, gives a value of ~32% (wt/wt) water of hydration (in good agreement with typical values). In the 1,000-mg/ml sample, this would leave only ~700 mg H_2O to form the structure stabilizing B-shell which would be slightly larger than a monolayer. On the other hand, the 500-mg/ml sample could form a B-shell approximately three water molecules thick. At lower temperatures the bound water joins in the ice state. This occurs with an increase in I_2 and decrease in τ_2. These changes resemble those of pure water which occur over a 20°C range

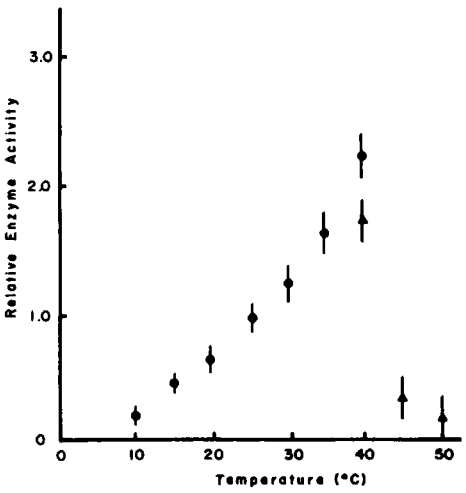

FIGURE 6

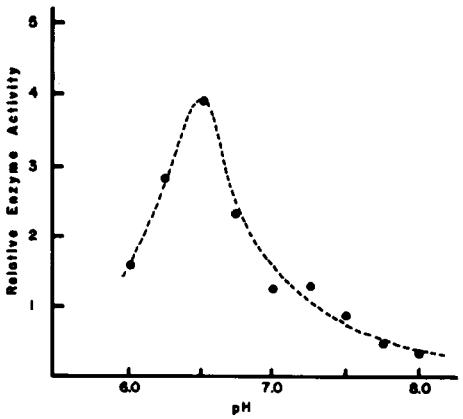

FIGURE 7

FIGURE 6 Temperature dependence of the esterase activity of BCA-B using *p*-nitrophenyl acetate as substrate. Activity is reported relative to 25°C (1.0). Dots indicate measurements in native protein. Samples indicated by Δ were preincubated for 60 min. No loss in activity was observed below 40°C.
FIGURE 7 pH activity profile of BCA-B (from Tallman et al., 1975).

centered about 0°C. In the 10 mg/ml sample, the transition is only slightly displaced from that of the pure water system. Water of hydration is likely to be bound in such geometrical configurations that it is incapable of ice formation without extensive rearrangements. The τ_2 values, at low temperatures, change more abruptly and at a lower temperature for the concentrated samples than for pure water and the 10-mg/ml sample. A freezing point depression of ~5°C for the 10-mg/ml sample and 15°C for the concentrated samples is observed.

Below 10°C the temperature dependence of τ_2 and I_2 primarily reflects ordering in A- and B-shell water for high protein concentrations, with bulk water effects becoming important at low concentrations, and no information on protein conformational changes may be obtained. Above 10°C, at high protein concentrations (Figs. 4 and 5), the temperature dependence of τ_2 and I_2 may be directly attributed to changes in protein free volume; τ_2 being a measure of average free volume region size and I_2 an indication of the number of such regions. This may be seen by comparison with the results shown for pure water. It may also be seen by comparison with results obtained with the highly stable protein ribonuclease (Glass et al., 1979). The temperature dependence of τ_2 and I_2 is virtually identical to that obtained here for BCA-B at temperatures below 10°C. Above 10°C, however, τ_2 and I_2 remain constant as a function of temperature, reflecting the high conformational stability of the protein.

The pH dependence of BCA-B was investigated to determine the nature and extent, if any, of conformational changes related to the pH-activity profile (Fig. 7). In this study, the 500-mg/ml sample was investigated over the pH range 6.0–7.6 The enzymatic activity (Tallman et al., 1975) is lowest at extremes of pH and reaches a maximum at pH 6.7. It appears from this study that the activity dependence on pH is not related to any significant conformational changes in the protein since we observe no changes in τ_2 or I_2 as a function of pH. In light of this, the strong pH dependence of carbonic anhydrase activity can be interpreted as being predominantly due to the ionization of an activity-linked group, with no resultant conformational change of the protein.

CONCLUDING REMARKS

The present study dealt with the evaluation of the positron annihilation lifetime technique as a probe of protein conformation. Combined temperature and concentration dependence studies of BCA-B clearly demonstrated that thermal denaturation is primarily associated with the formation of large voids or cavities in the structure and is accompanied by the overall expansion of the protein. After denaturation, the protein is not in a completely random state but rather an inactive expanded conformation.

A pH dependence study over the physiological pH range showed no change in τ_2 between pH 6.7 and 7.0. Within this range an activity-linked group undergoes a catalytically important ionization. It appears that the role of this ionization in the mechanism is predominantly chemical and that only a very subtle, if any, conformational role is involved.

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