NMR studies of fluorophenylalanine-containing carbonic anhydrase

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Rabbits ingesting 4-fluorophenylalanine are known to incorporate small amounts of this fluorinated amino acid into their proteins. Carbonic anhydrase I isolated from the erythrocytes of animals so maintained exhibits a well-resolved fluorine NMR signal for each phenylalanine in the sequence. The chemical shifts of most of these signals respond to the binding of inhibitors, suggesting that most if not all of the tertiary structure of the enzyme adjusts to the presence of inhibitors at the the active site.

fluorine NMR Carbonic anhydrase Enzyme inhibition Protein structure

1. INTRODUCTION

Carbonic anhydrase is an enzyme which catalyzes the reversible hydration of carbon dioxide. It is found in virtually all tissues and often in several isozymic forms within a given organism [1,2]. The most abundant isozymes are strongly inhibited by a variety of anions [3] and sulfonamide derivatives are also excellent competitive inhibitors [4]. Clinically, carbonic anhydrase inhibitors are effective in management of glaucoma and edema.

¹H-NMR studies of carbonic anhydrase have been largely limited to examination of the titration behavior of histidine and amide N-H resonances [5,6]. The aromatic proton region, although exhibiting considerable fine structure [6], contains 120–130 signals from the approx. 2 dozen aromatic amino acids present in the sequence as well as various amide N-H signals, and has not been especially useful in probing structural features of the enzyme. Crystallographic studies show that phenylalanine is distributed throughout the tertiary structure of the human enzyme, with a tendency to be found in aromatic clusters [7,8].

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The number and position of phenylalanine residues are highly conserved in the carbonic anhydrase I and II isozymes of mammals.

Westhead and Boyer [9] have shown that 4-fluorophenylalanine present in the diet of the rabbit is incorporated into proteins of muscle, blood and liver. It has previously been determined that hemoglobin isolated from animals maintained in this way contains enough fluorophenylalanine that well-resolved fluorine NMR spectra can be obtained in several hours using a sample 2–3 mM in protein [10,11]. We now report that carbonic anhydrase I isolated from erythrocytes of such rabbits contains detectable amounts of 4-fluorophenylalanine and that fluorine NMR spectra of this protein are highly responsive to the presence of inhibitors.

2. MATERIALS AND METHODS

New Zealand White rabbits were fed a diet containing 0.3% (by wt) of DL-4-fluorophenylalanine (Calbiochem) and bled at biweekly intervals. Hemolysate was prepared as described [9].

The carbonic anhydrase isozymes were isolated by the affinity gel procedure of Osborne and Tashian [12] as modified by Khalifah et al. [13]. The isozymes were further purified by ion-exchange chromatography on DEAE-Sephadex by a procedure similar to that of MacIntosh [14]. Approx. 400 ml hemolysate, collected over a series of bleedings, afforded about 20 mg enzyme which was used for all of the experiments reported here. The enzyme (pI 7.9) was 90% pure by isoelectric focusing, displaying an impurity at pI 8.1.

Amino acid composition, including the amount of fluorophenylalanine present, was assayed by an HPLC method [15].

Esterase activity of fluorinated and non-fluorinated carbonic anhydrases was measured using p-nitrophenyl acetate [16].

Fluorine NMR spectra were obtained at 282.3 MHz on a Nicolet NT-300 spectrometer using a pulse width of 15 μ s (68° flip-angle) and an acquisition time of 540 ms without a relaxation delay. FIDs were usually multiplied by a double exponential function to enhance the resolution of the resulting spectra.

3. RESULTS AND DISCUSSION

Amino acid analysis of hydrolysates of rabbit carbonic anhydrase I indicated the presence of 11 phenylalanines, a result consonant with the sequence recently determined for this enzyme [17,18]. An important difference between the results of Konialis et al. [18] and the partial sequence reported previously [17] is the replacement of a lysine by a phenylalanine at position 47, resulting in the total of 11 Phe residues.

Amino acid analysis also showed the presence of approx. 0.5 mol 4-fluorophenylalanine per mol protein. That is, each molecule of the enzyme has, on average, not more than one fluorophenylalanine residue. Esterase activity of enzyme obtained from control rabbits was found to be in good agreement with literature $(k_{cat}/K_m = 300 \text{ M}^{-1} \cdot \text{s}^{-1})$, this work; 299 $\text{M}^{-1} \cdot \text{s}^{-1}$ [15]). Activity of the fluorine-containing enzyme was somewhat higher $(390 \text{ M}^{-1} \cdot \text{s}^{-1})$ initially but declined to 220 $\text{M}^{-1} \cdot \text{s}^{-1}$ by the end of the series of experiments described here. Although these observations suggest that fluorine substitution enhances the activity of the enzyme and decreases its stability, these conclusions must remain very tentative

until additional studies are completed as more enzyme becomes available.

Fluorine **NMR** spectra of 4-fluorophenylalanine-containing rabbit carbonic anhydrase I show a series of well-resolved signals (fig.1). Some variation in signal intensity is expected because of differences in spin-lattice relaxation times (0.2-0.5 s) of the various signals and the rapid pulsing conditions used to obtain the spectra. Nevertheless, the spectra are consistent with the presence of 11 signals of roughly equal intensity in each spectrum. (Two overlapping resonances appear at approx. -0.5 and -3 ppm in some cases.) Thus, fluorophenylalanine apparently is incorporated at random at Phe locations throughout the protein rather than being localized at only a few. Zero on the chemical shift scale in fig.1 is set where free 4-fluorophenylalanine is expected and the

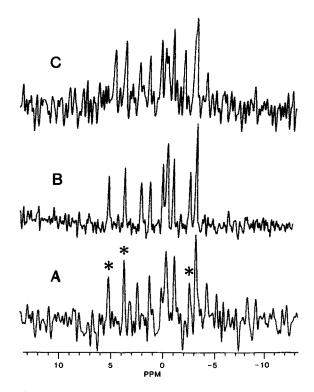


Fig.1. Fluorine NMR spectra of fluorophenylalanine-containing rabbit carbonic anhydrase I at 282.3 MHz. Each sample contained 0.05 M Tris-sulfate and 5% D₂O, pH 7.5. The sample temperature was 25°C in each case. (A) Native enzyme (0.24 mM), (B) enzyme (0.5 mM) with sodium azide (5 mM), (C) enzyme (0.5 mM) with p-toluenesulfonamide (0.7 mM).

wide range of chemical shifts on both sides of this position reflect in some way details of the local environment of each fluorophenylalanine.

most extensively studied form fluorinated rabbit carbonic anhydrase I was a sample inhibited with an excess of azide (fig.1B). A large excess of azide was used to ensure a homogeneous form of the enzyme since many anions, including buffer salts, bind to carbonic anhydrases [19]. Removal of the azide inhibitor from the sample resulted in the spectrum shown in fig.1A. While similar to the spectrum of the azideinhibited protein, small shifts of most resonances take place while an additional, smaller resonance appears upfield (~4.2 ppm). This resonance is similar in position to the signal at highest field found with the azide-inhibited material at high pH and may indicate that a phenylalanine in the uninhibited enzyme is in slow conformational exchange between two (or more) environments characterized by unique chemical shifts.

A slight molar excess of p-toluenesulfonamide was added to the sample used to obtain fig.1B. This sample produced spectrum C in fig.1. Presuming a dissociation constant similar to the values found for the bovine and human enzymes, under the experimental conditions, essentially all of the enzyme should have inhibitor bound to it. Carbonic anhydrase with the sulfonamide bound shows a shift of most fluorine resonances when compared to the spectrum of the uninhibited enzyme. Human carbonic anhydrase I has a phenylalanine at position 91, near the sulfonamide-binding site. However, this amino acid is replaced by serine in the rabbit and, thus, changes at this residue are not associated with the chemical shift changes seen in trace C. The resonances marked (*) are among the most strongly affected by sulfonamide binding. The sulfonamide-binding site is presumably near the metal atom of the enzyme [20], and these signals are broadened beyond detection when the zinc at the active site of the fluorinated carbonic anhydrase is replaced by cobalt (not shown). The phenylalanine residues represented by these signals, therefore, must be reasonably close to the active site.

Fluorine chemical shifts in proteins are likely dominated by van der Waals interactions and, thus, are dependent on the number of atoms in the immediate vicinity of a fluorine nucleus and distances to those atoms [21,22]. The chemical shift differences between spectra of fluorophenylalanine-containing carbonic anhydrase I in the presence and absence of inhibitors indicate that the tertiary structure changes upon binding of inhibitors. Phe/fluorophenylalanine is found throughout the protein structure and the shift changes, while most dramatic in the vicinity of the active site, are not exclusively localized there. That is, the entire protein responds conformationally to binding of small molecules at the active site.

Similar experiments with other isozymes from the rabbit and enzymes from other species are underway and it will be of interest to determine the generality of these conclusions.

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