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# A Comparative Study of Mammalian Erythrocyte Carbonic Anhydrases Employing Spin-Labeled Analogues of Inhibitory Sulfonamides

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SUMMARY

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The topography of the active sites of rhesus carbonic anhydrases I and II, rabbit carbonic anhydrase I, sheep carbonic anhydrase II, and dog carbonic anhydrase I has been studied with the aid of spin-labeled analogues of acetazolamide and sulfanilamide. Electron spin resonance measurements indicated that the pyrrolidine ring of 2,2,5,5-tetramethyl-3-[(*p*-sulfamoylphenyl)carbamoyl]-1-pyrrolidinyloxyl became highly immobilized when this label bound to the active site of rhesus carbonic anhydrase I. As the chain length between the aromatic and pyrrolidine rings was increased, the mobility of the nitroxide group of the enzyme-bound inhibitor progressively increased, until with 2,2,5,5-tetramethyl-3-({[(*p*-sulfamoylphenyl)carbamoyl]methyl}carbamoyl)-1-pyrrolidinyloxyl there was only minimal interaction between the heterocyclic ring and the active site of rhesus carbonic anhydrase I. These findings suggest that the active site of rhesus carbonic anhydrase I is a cleft about 14 A deep. Similar experiments indicated that the topography of the active sites of rhesus carbonic anhydrase II, dog carbonic anhydrase I, and sheep carbonic anhydrase II were similar to that of rhesus carbonic anhydrase I, while the active site of rabbit carbonic anhydrase was somewhat deeper. Spin-labeled

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inhibitor 2,2,6,6-tetramethyl-4-(*p*-sulfamoylbenzamide)piperidinooxyl became highly immobilized on binding to type II (high-activity) carbonic anhydrase but exhibited isotropic motion at the active sites of type I (low-activity) isozymes. An attempt is made to explain these results in terms of the three-dimensional structure of the active sites of human carbonic anhydrases I and II.

## INTRODUCTION

Erythrocyte carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) is a zinc-containing metalloenzyme that reversibly catalyzes the hydration of $CO_2$ (1, 2). This enzyme is specifically inhibited by aromatic sulfonamides that have the general formula $ArSO_2NH_2$, where Ar is either homocylic or heterocyclic (1). The erythrocytes from many mammalian species contain two distinct isozymes of carbonic anhydrase, designated types I and II, which are known to be the products of independent gene loci (3, 4). While these isozymes have been found to have quite similar molecular structures, they differ from each other in that type I isozymes have a much higher hydro-lyase turnover number. Type I isozymes have been isolated from the erythrocytes of humans (5), rabbit (8), rhesus monkey,[3] horse (7), and dog (6, 8), while type II isozymes have been found in red cells from humans (5), rabbit (8), dog (6, 8), sheep (9), rhesus monkey,[3] ox (10), and horse (7). The complete amino acid sequences of human carbonic anhydrase I, human carbonic anhydrase II (9–14), bovine carbonic anhydrase II (4, 15, 16), and sheep carbonic anhydrase II (9) have been elucidated. In addition, Kannan and his co-workers have determined the crystal structure of both human isozymes to a 2-A resolution (17–20). These studies show that the active site of the human carbonic anhydrases consists of a conical cavity at the bottom of which is a single zinc atom. The active site cavity of the human isozymes can be divided into a hydrophobic and a hydrophilic half, partitioned through the zinc atom (20). The amino acid residues present in these two halves are listed in Table 1. When the sulfonamide inhibitor acetazolamide binds to human carbonic anhydrase II, the sulfonamide nitrogen (or oxygen) atom is directly coordinated to the zinc atom. The distance between the sulfur and zinc atoms is 3.6 A (21).

Spin labels are stable free radicals that can be used as probes for biologically important macromolecules such as proteins, nucleic acids, and membranes (22–24). In previous studies we have shown that spin-labeled analogues of sulfonamide inhibitors containing the nitroxide radical (I–VII, Table 2) can be used as probes for the active site of erythrocyte carbonic anhydrases (24–28). For example, using a series of spin-labeled sulfonamides (I–VI) in which the distance between the aromatic group and the pyrrolidine ring that bears the nitroxide function was gradually increased, it was possible to estimate that the depth of the active site of human carbonic anhydrase II is about 14 A (26). This value is in good agreement with crystallographic data (19, 21), suggesting that the crystal and solution conformations of this enzyme are similar. Studies of other mammalian erythrocyte carbonic anhydrases indicated that their active sites had approximately the same molecular dimensions as human carbonic anhydrase II. The only difference between type I and II isozymes which was detected by these previous measurements was that spin labels III–V were more immobilized when bound to type I isozymes. On the basis of this observation, it was suggested that the active sites of type I isozymes might be narrower or perhaps slightly deeper than the active sites of the higher-activity, type II isozymes (27, 28).

In the present study spin labels I–VI have been employed to study the topography of the active sites of the following erythrocyte carbonic anhydrases: rhesus monkey I and II, dog I, rabbit I, and sheep II. The interaction of spin label VII with these carbonic anhydrases as well as with human carbonic anhydrases I and II and bovine carbonic anhydrase II has also been examined. The results indicate that spin

[3] R. J. Tanis, unpublished results.

TABLE 1

*Exposed side chains in active site of human carbonic anhydrases I and II*[a]

| Residue No.[b] | Polar amino acid residues | | | | Residue No. | Nonpolar amino acid residues | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Human carbonic anhydrase I | | Human carbonic anhydrase II | | | Human carbonic anhydrase I | | Human carbonic anhydrase II | |
| | Amino acid | Distance from α-carbon to zinc atom | Amino acid | Distance from α-carbon to zinc atom | | Amino acid | Distance from α-carbon to zinc atom | Amino acid | Distance from α-carbon to zinc atom |
| 6 | Tyr | 13.61 | Tyr | 13.73 | 64 | *Ser* | 9.50 | *Ala* | 9.33 |
| 60 | Asn | 14.55 | Asn | 14.48 | 90 | *Phe* | 12.34 | *Ile* | 12.44 |
| 63 | His | 10.53 | His | 10.80 | 120 | *Ala* | 7.94 | *Val* | 7.88 |
| 66 | *His* | 10.45 | *Asn* | 10.40 | 129 | *Leu* | 15.67 | *Phe* | 14.90 |
| 68 | *Asn* | 13.11 | *Glu* | 14.00 | 133 | *Ala* | 13.31 | *Val* | 14.15 |
| 91 | Gln | 9.06 | Gln | 10.23 | 139 | Leu | 11.66 | Leu | 11.44 |
| 93 | His[c] | 5.03 | His[c] | 6.64 | 141 | Val | 8.28 | Val | 7.29 |
| 95 | His[c] | 6.07 | His[c] | 6.81 | 143 | Gly | 8.64 | Gly | 7.88 |
| 118 | His[c] | 4.99 | His[c] | 5.05 | 199 | Pro | 10.79 | Pro | 10.66 |
| 197 | Thr | 6.39 | Thr | 6.17 | 200 | Pro | 12.02 | Pro | 11.79 |
| 198 | *His* | 8.73 | *Thr* | 7.65 | 202 | *Tyr* | 12.79 | *Leu* | 13.15 |
| | | | | | 204 | *Ser* | 13.20 | *Cys*[d] | 12.93 |
| | | | | | 205 | Val | 10.67 | Val | 10.90 |
| | | | | | 209 | *Ile* | 11.05 | *Val* | 12.12 |

[a] Adapted from ref. 20. Residues which are different in the two isozymes have been italicized.

[b] Based on the sequence for human carbonic anhydrase II.

[c] The zinc atom is bonded to the $N^{\epsilon_2}$ nitrogen of His-93 and His-95 and to the $N^{\delta_1}$ nitrogen of His-118.

[d] The Cys-204 residue is not exposed in human carbonic anhydrase II, but the corresponding Ser-204 residue in human carbonic anhydrase I is exposed.

label VII can differentiate between type I and II isozymes. Finally, an attempt has been made to explain the observed differences in the interaction of spin labels I–VII with human carbonic anhydrases I and II in terms of the crystallographic structures of these isozymes.

## MATERIALS AND METHODS

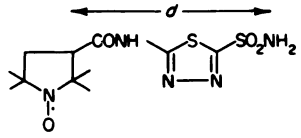
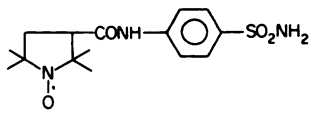
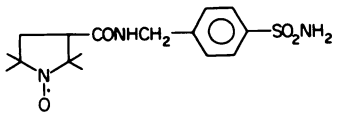
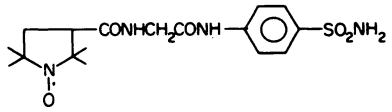
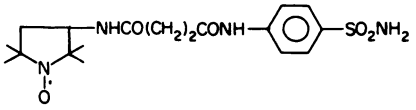
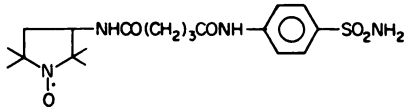
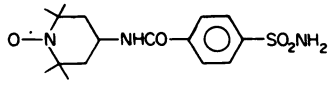
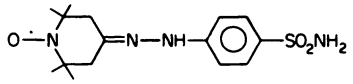
Human erythrocyte carbonic anhydrases I and II were prepared from outdated blood bank blood by the method of Armstrong and co-wrokers (5). Bovine carbonic anhydrase II was purchased from Worthington Biochemicals and purified by DEAE-cellulose chromatography (10). Sheep carbonic anhydrase II was isolated by previously reported procedures (9). Rhesus carbonic anhydrases I and II, rabbit carbonic anhydrase I, and dog carbonic anhydrase I were prepared by an affinity column procedure similar to that described by Osborne and Tashian (29). The type I designation for the dog[3] and rabbit (8) carbonic anhydrases was made on the basis of both amino acid sequences and enzymatic activities. The concentrations of the carbonic anhydrases were determined spectrophotometrically using the following $A^{1\%}_{1\,cm}$ values at 280 nm: human carbonic anhydrase I, 16.3; human carbonic anhydrase II, 17.8 (5); bovine carbonic anhydrase II, 18 (10); sheep carbonic anhydrase II, 16.1 (3); rhesus carbonic anhydrase I,[3] 16.2; rhesus carbonic anhydrase II,[3] 17.8; dog carbonic anhydrase I, 17;[3] and rabbit carbonic anhydrase I, 17.0 (8). The spin-labeled sulfonamides (I–VII) were prepared by previously reported procedures (26, 30). All other chemicals were of reagent grade and were used without further purification.

The ESR spectra were recorded at 25° with a Varian E-4 spectrometer equipped with a quartz aqueous sample cell as previously described (26).

The esterase activity of human carbonic anhydrases I and II was measured by the spectrophotometric technique of Armstrong and co-workers (5), using *p*-nitro-

TABLE 2

*Structures of sulfonamide spin labels*

| Spin Label | Structure | *d* |
|---|---|---|
| | | *A* |
| I |  | 7.44 |
| II |  | 8.16 |
| III |  | 8.88 |
| IV |  | 11.28 |
| V |  | 12.72 |
| VI |  | 14.72 |
| VII |  | 7.86 |
| VIII |  | |

phenylacetate as the substrate. The $K_I$ value for spin label VII was calculated by the method of Armstrong *et al.* (5).

The molecular distances for human carbonic anhydrases I (B) and II (C) (Table 1 and Fig. 6) were determined with the aid of the XRAY program run on a Digital Equipment Corporation PDP10 computer (31). The molecular dimensions of spin labels I-VII (Table 2 and Fig. 6) were determined with the aid of the same programs by constructing the labels from the known crystal coordinates of molecular fragments. Where the crystal coordinates were not available, Corey-Pauling-Koltun space-filling models were used instead.

The rotational correlation time $\tau_c$ for the spin labels was calculated from the relative heights of the three hyperfine lines and the width of the center line, using the equations described by Smith (32). The following crystal parameters were employed for spin labels I-VI: $T_{zz}$ = 31 G, $T_{xx}$ = 4.7 G, and $T_{yy}$ = 4.7 G (33). For label VII the

corresponding values were $T_{zz}$ = 31 G, $T_{xx}$ = 5.2 G, and $T_{yy}$ = 5.2 G (33).

## RESULTS

*Effect of molecular motion on ESR spectrum of nitroxide radical.* In dilute aqueous solution at room temperature, the ESR spectra of the spin-labeled sulfonamides consist of three sharp lines with a hyperfine splitting of approximately 16 G (26). When the molecular motion of the sulfonamide labels is slowed by increasing solvent viscosity, the hyperfine lines appear to exhibit unequal broadening (Fig. 1A and B). The limiting line shape (Fig. 1E), or "rigid glass" spectrum, is characterized by the appearance of distinct high- and low-field extrema separated by approximately 64 G. The molecular motion of a spin label may be characterized by its rotational correlation time $\tau_c$, which operationally may be defined as the time required for the label to rotate through one radian. For weakly immobilized spin labels ($\tau_c$ = $10^{-11}$-$10^{-9}$ sec), which give isotropic spectra (Fig. 1A and B), $\tau_c$ can be estimated directly from the relative line heights and the width of the center line (32). While it is also possible to calculate $\tau_c$ from anisotropic spectra (Fig. 1D and E), which are characteristic of highly immobilized labels ($\tau_c >> 10^{-8}$ sec), this cannot normally be accomplished without resorting to computer simulation. The spectra from highly immobilized labels may, however, be characterized by their maximal hyperfine splitting ($2T_{\parallel}$), which is the distance between the high- and low-field extrema (Fig. 1E).

*Interaction of spin-labeled sulfonamides I-VI with carbonic anhydrase.* In the presence of rhesus carbonic anhydrase I, the ESR spectrum of spin label I became broad and highly asymmetrical, with a maximal hyperfine splitting ($2T_{\parallel}$) of 62 G (Fig. 2A and Table 3). This observation suggests that when label I binds to the rhesus type I isozyme, the pyrrolidine ring becomes highly immobilized. Similar results were obtained for spin label II, except that for

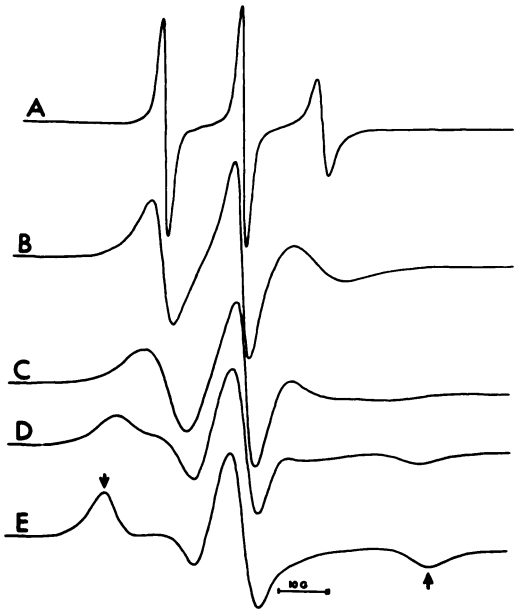



FIG. 1. *ESR spectrum of sulfonamide III (0.1 mM) dissolved in glycerol*

A. 60°. B. 30°. C. 20°. D. 10°. E. 0°. The arrows indicate the position of the high- (left) and low- (right) field peaks characteristic of a highly immobilized nitroxide radical.

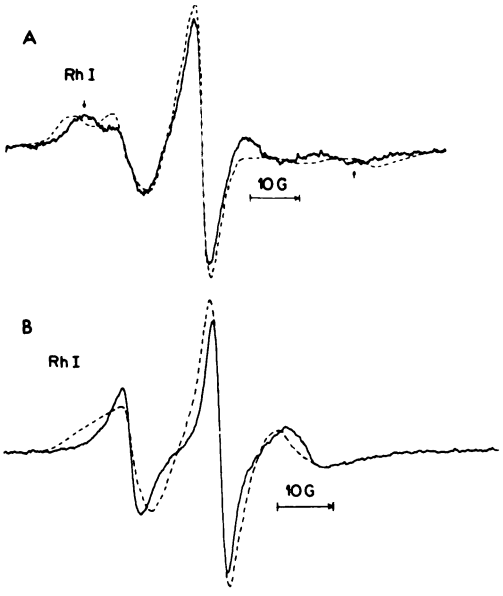



FIG. 2. *ESR spectra of sulfonamides I and II (A) and III and IV (B) with rhesus carbonic anhydrase I*

- - -, sulfonamides I (A) and III (B); ——, sulfonamides II (A) and IV (B), all at 20 μM; carbonic anhydrase I, 53 μM, in 0.1 M sodium phosphate buffer (pH 7.4). The arrows (A) indicate the positions of the high- (left) and low- (right) field peaks characteristic of highly immobilized spin label II.

TABLE 3

*ESR spectral parameters of sulfonamide spin labels bound to carbonic anhydrases from various mammalian species*

| Spin label | $d^a$ | Rhesus I | | Rhesus II | | Dog I | | Rabbit I | | Sheep II | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $\tau_c$ | $2T_{\parallel}$ | $\tau_c$ | $2T_{\parallel}$ | $\tau_c$ | $2T_{\parallel}$ | $\tau_c$ | $2T_{\parallel}$ | $\tau_c$ | $2T_{\parallel}$ |
| | *A* | *nsec* | *G* | *nsec* | *G* | *nsec* | *G* | *nsec* | *G* | *nsec* | *G* |
| I | 7.44 | | 62.0 | | 58.0 | | 59.5 | | 59.0 | | 60.5 |
| II | 8.16 | | 55.0 | | 58.5 | | 52.5 | | 55.0 | | 57.5 |
| III | 8.88 | 5.90 | | 2.72 | | 4.57 | | | 59.0 | 5.46 | |
| IV | 11.28 | 2.84 | | 1.91 | | 2.38 | | 3.59 | | 4.65 | |
| V | 12.72 | 3.35 | | 1.98 | | 2.18 | | 3.54 | | 2.62 | |
| VI | 14.72 | 2.35 | | 2.88 | | 2.54 | | 1.91 | | 3.56 | |

[a] See Table 2.

this label the value of $2T_{\parallel}$ was 55 G. Thus it would appear that the pyrrolidine ring of spin label II has greater mobility at the active site of rhesus carbonic anhydrase than does the pyrrolidine ring of spin label I. The corresponding $2T_{\parallel}$ values for spin labels I and II bound to rhesus carbonic anhydrase II were 58 and 58.5 G, respectively (Table 3). This would suggest that the pyrrolidine rings of spin labels I and II have approximately the same mobility at the active site of rhesus carbonic anhydrase II. The insertion of a single methylene group between the aromatic ring and the amino group of label II to give spin label III (Table 2) caused a dramatic increase in the mobility of the nitroxide group at the active site of both rhesus isozymes (Figs. 2B and 3A and Table 3). The reappearance of the three-line spectrum suggests that the motion of the pyrrolidine group in spin label III is isotropic. A comparison of the $\tau_c$ values for the complexes between spin label III and the two rhesus isozymes (Fig. 3A) indicates that the molecular motion of the pyrrolidine ring of label III was twice as rapid at the active site of the rhesus II isozyme. When the distance between the benzene and pyrrolidine rings was increased again to give label IV (Table 2), there was a further increase in the molecular motion of the pyrrolidine ring at the active site of rhesus carbonic anhydrase I (Table 3 and Figs. 2B and 3A). While spin label IV also showed greater molecular motion than label III on binding to rhesus carbonic anhydrase II, the difference was much smaller than observed for isozyme I. When compared with

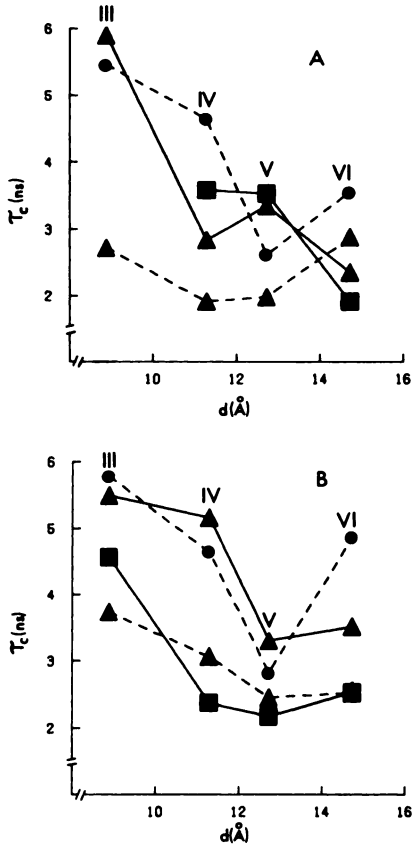



FIG. 3. *Rotational correlation times. $\tau_c$ (nanoseconds), of spin labels III-VI bound to various mammalian carbonic anhydrases*

A. ▲——▲, rhesus carbonic anhydrase I; ▲- - -▲, rhesus carbonic anhydrase II; ●- - -●, sheep carbonic anhydrase II; ■——■, rabbit carbonic anhydrase I. B. ▲——▲, human carbonic anhydrase I; ▲- - -▲, human carbonic anhydrase II; ●- - -●, bovine carbonic anhydrase II; ■——■, dog carbonic anhydrase I. For the definition of *d*, see Table 2.

label IV, the mobility of the pyrrolidine ring of label V was slightly less at the active site of rhesus carbonic anhydrase I, while the pyrrolidine ring of label VI was somewhat more mobile (Figs. 3A and 4A). The pyrrolidine ring of label V had the same mobility as label IV at the active site of rhesus carbonic anhydrase II, while label VI exhibited less motion.

The ESR spectral parameters for the binding of labels I–VI to dog and rabbit carbonic anhydrases I and sheep carbonic anhydrase II are shown in Table 3 and Fig. 3. In each case the maximal hyperfine splittings ($2T_{\parallel}$) suggest that spin label I is more immobilized than spin label II. The rabbit carbonic anhydrase I is somewhat unusual, in that spin label III is highly immobilized by this enzyme; the same label exhibits isotropic motion in the active sites of the other carbonic anhydrases. The ESR spectrum of spin label II bound to rabbit carbonic anhydrase I contains two components (Fig. 4B). The low-field line 1 (Fig. 4B) and high-field line 4 belong to a population of highly immobilized labels, while lines 2 and 3 belong to a second population of more mobile labels. The center line contains contributions from both populations.

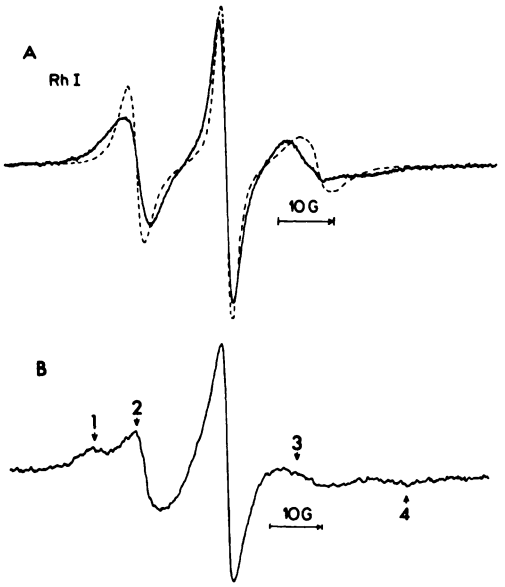



FIG. 4. *ESR spectra of sulfonamides V (——) and VI (- - -) bound to rhesus carbonic anhydrase I (53 μM) (A) and of sulfonamide II bound to rabbit carbonic anhydrase I (76 μM) (B)*

The concentration of each sulfonamide was 20 μM in 0.1 M sodium phosphate buffer (pH 7.4).

*Interaction of spin label VII with carbonic anhydrase*. In our original studies of bovine carbonic anhydrase II, we employed the piperidine spin label VII, which became highly immobilized when bound to the active site of this enzyme. However, when label VII was used to examine the active sites of the human isozymes, it was found that, while this label became highly immobilized on binding to isozyme II, it exhibited isotropic motion at the active site of human carbonic anhydrase I (Table 4 and Fig. 5). This rather puzzling finding led to the abandonment of piperidine label VII for the topographical studies. However, since bovine carbonic anhydrase II and human carbonic anhydrase II are both high-activity isozymes while human carbonic anhydrase I is a low-activity isozyme, it appeared that spin label VII might be capable of differentiating between these two forms. The results shown in Table 4 indicate that, without exception, spin label VII is more mobile at the active site of the low-activity, type I isozymes than it is at the active site of the higher-activity, type II isozymes. It is of interest that the $K_I$ values for inhibition of the esterase activity of human isozymes I and II by label VI were 93 nM and 3.7 nM, respectively. Thus the stronger interaction between spin label VII and the active site of human carbonic anhydrase II, which is suggested by the ESR measurements, is

TABLE 4

*ESR spectral parameters of spin label VII bound to erythrocyte carbonic anhydrases from various mammalian species*

| Carbonic anhydrase | $\tau_c$ | $2T_{\parallel}$ |
|---|---|---|
| | *nsec* | *G* |
| Human I | 3.85 | |
| Human II | | 59.3 |
| Bovine II | | 56.0 |
| Rhesus I | 4.35 | |
| Rhesus II | | 58.5 |
| Dog I | 4.41 | |
| Rabbit I | 2.77 | |
| Sheep II | | 56.5 |

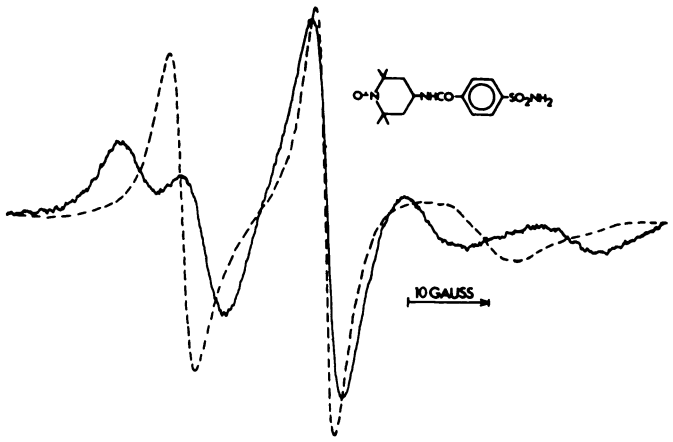



FIG. 5. *ESR spectra of sulfonamide VII (20 μM) bound to human carbonic anhydrases I (- - -) and II (——) (262 μM) in the presence of 0.1 M sodium phosphate buffer (pH 7.4)*

also supported by the enzymatic data, which demonstrate that VII is 25 times more active as an inhibitor of this isozyme.

## DISCUSSION

The X-ray crystallographic data of Kannan and co-workers (20) show quite clearly that the active site of human carbonic anhydrase is conical in shape and that the zinc atom is located at the bottom of this site. While there are differences in the amino acid residues at the active sites of the human carbonic anhydrases, it will be seen from Table 1 that, as judged by the relative distances between the zinc atom and the α-carbon atoms of the active site amino acids, the topography of the active sites of these two isozymes is quite similar. There are a total of 11 amino acid residues which differ at the active sites of the human isozymes (Table 1). The spatial relationships between these amino acids and the zinc atom are shown diagrammatically in Fig. 6. The important differences between the active sites of the human isozymes have been summarized by Nostrand *et al.* (20). The hydrophobic part of the cavity in human carbonic anhydrase I contains a polar residue, serine 204, which replaces cysteine 204 in human carbonic anhydrase II. In human carbonic anhydrase I, tyrosine 202, which is homologous with leucine 202 in human carbonic anhydrase II, is oriented away from the cavity, leaving this region somewhat more open in this isozyme. Two other important changes are the replacement of isoleucine 90 and phenylalanine 129 in human carbonic anhydrase II by phenylalanine 90 and leucine 129 in human carbonic anhydrase I. These differences, together with the substitution of valines 120 and 133 in human carbonic anhydrase II for alanines 120 and 133 in human carbonic anhydrase I, change both the character and content of the hydrophobic portion of the cavity. There are also changes in the hydrophilic half of the cavity (Table 1 and Fig. 6), but these seem less likely to affect the binding of the hydrophobic spin labels.

One finding which emerges from an examination of the X-ray crystallographic data is that the active sites of both human isozymes are quite wide. For example, the distance between the α-carbon atoms of glutamic acid 68 and leucine 202, two amino acids which appear to be at or near the surface of human carbonic anhydrase II but on opposite sides of the active site cavity, is 22.1 A. The distance between the corresponding α-carbon atoms of asparagine 68 and tyrosine 202 in human carbonic anhydrase I is 20.2 A. Since the molecular dimensions of the pyrrolidine ring that bears the nitroxide function in labels I–VI are on the order of 4 A, it seems unlikely that the dramatic decrease in the molecular motion observed when labels I and II bind to the human isozymes (26) can

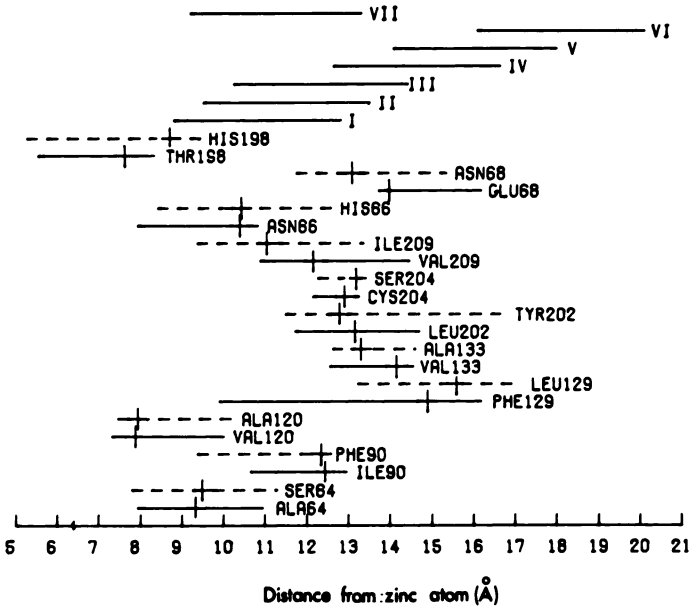



FIG. 6. *Spatial relationship between zinc atom and atoms of amino acid residues at active sites of human carbonic anhydrases I (- - -) and II (——)*

Each line represents the range of atomic distances between the zinc atom and the individual atoms of the various amino acids as determined from the X-ray crystal coordinates for each isozyme. The vertical lines represent the positions of the α-carbon atoms. Also shown are the estimated ranges of atomic distances between the zinc atom and the individual atoms of the pyrrolidine or piperidine rings of labels I-VII. The distances for the spin labels were calculated from the X-ray crystal coordinates for acetazolamide (34), sulfanilamide (35), and the pyrrolidine (36) or piperidine (37) ring systems, together with Corey-Pauling-Koltun models when the crystal data were unavailable.

be explained solely in terms of steric hindrance. The finding by Chen and Kernohan (38) that, in the presence of bovine carbonic anhydrase II, the quantum yield of 1-dimethylaminonaphthalene-5-sulfonamide increases dramatically while its fluorescence emission maximum moves to shorter wavelengths suggests that this sulfonamide interacts primarily with the hydrophobic half of the active site of this enzyme. It seems not unreasonable, therefore, to postulate that hydrophobic interactions between the pyrrolidine ring and the side chains of the hydrophobic amino acids may be responsible for the strong immobilization of this group when spin labels I and II bind to the active site of the human carbonic anhydrases.

It can be clearly seen from Fig. 3B that for human carbonic anhydrase I a minimum $\tau_c$ value is first reached with spin label V. X-ray crystallographic studies of human carbonic anhydrase II at 2-A resolution have shown that the average distance between the zinc atom of this isozyme and the sulfur atom of three sulfonamide inhibitors is 3 A (21). For acetazolamide, X-ray crystallographic measurements (34) indicated that the nitrogen-sulfur distance is 1.6 A. If the zinc, nitrogen, and sulfur atoms lie in a straight line, the zinc-nitrogen distance will be 1.4 A. With the aid of X-ray crystallographic coordinates for acetazolamide (34) and the pyrrolidine ring (36), it is possible to estimate that the distance from the nitrogen atom of the sulfonamide group to the carbon at position 3 of the pyrrolidine ring of spin label I in an extended conformation is 7.44 A, whereas the distance from the sulfonamide nitrogen atom to the nitroxide oxygen atom is 11.44 A. If the estimated distance between the zinc atom and the nitrogen atom is added, the total distance be-

tween the zinc atom and carbon atom 3 of the pyrrolidine ring in spin label II will be 8.84 A (7.44 + 1.4), while the distance between the zinc and the nitroxide oxygen will be 12.84 A (11.44 + 1.4). The corresponding distances for labels II–VI, calculated with the aid of the X-ray coordinates for sulfanilamide (35) and Corey-Pauling-Koltun models, are shown in Fig. 6. The distance between the zinc atom and carbon 3 of the pyrrolidine ring of spin label V bound to carbonic anhydrase would be 14.12 A if the label bound in an extended conformation. This distance is an entirely reasonable upper-limit estimate for the depth of the active site of human carbonic anhydrase I (cf. Table 1 and Fig. 6). The observation that the pyrrolidine rings of spin labels IV and V have almost the same mobility when bound to human carbonic anhydrase II suggests that the active site of this enzyme may be somewhat shallower than that of isozyme I. An examination of Fig. 6 shows that tyrosine 202, and perhaps leucine 129, in human carbonic anhydrase I may be responsible for the greater immobilization of spin label IV at the active site of this enzyme. The good agreement between the data obtained from the spin label "molecular dipstick" approach and the crystallographic structure of the human isozymes provides convincing evidence that the crystal and solution conformations of these enzymes are the same.

Spin label IV exhibited a high degree of mobility when bound to rhesus carbonic anhydrases I and II (Fig. 3A) and dog carbonic anhydrase I (Fig. 3B). This would suggest that active sites of these enzymes are also shallower than that of human carbonic anhydrase I. The strong immobilization of label III at the site of rabbit carbonic anhydrase I (Table 3) and the observation that maximal mobility of the pyrrolidine ring was not reached until label VI (Fig. 3A) would indicate that the active site of this enzyme is, on the other hand, deeper than that of the human carbonic anhydrase I. The gradual increase in the mobility of the pyrrolidine ring when progressing from label I to label VI provides some evidence that the active sites of all the isozymes studied in this investigation are conical rather than slitlike.

An examination of the data for the binding of spin label VII to type I and II isozymes from different species shows quite clearly (Fig. 5 and Table 4) that the piperidine ring of this label is much more mobile at the active site of type I isozymes than it is at the active site of type II isozymes. If the assumption is made that these findings are due to the existence of different amino acid side chains at the active site, it can be seen from Fig. 6 that for the human isozymes there are several possibilities which might explain the results on a molecular basis. The most obvious candidate is cysteine 204 in human isozyme II, which is replaced by serine 204 in isozyme I. This change introduces a polar amino acid into the hydrophobic portion of the active site. However, sheep carbonic anhydrase II also has a serine at position 204, and yet spin label VII is still highly immobilized at the active site of this enzyme (Table 4). If serine 204 occupies the same position in sheep carbonic anhydrase II as cysteine 204 does in human carbonic anhydrase I, this would tend to rule out the possibility that this amino acid is responsible for the increased mobility of spin label VII at the active site of human carbonic anhydrase I. Another important difference is the substitution of leucine 129 in human carbonic anhydrase I for phenylalanine 129 in human carbonic anhydrase II. This substitution places an aromatic ring system at a point in the active site of human carbonic anhydrase II where it could possibly interact with the piperidine ring of label VII.

At the present time it is difficult to provide an explanation for the difference in the interaction of spin labels I and VII with human carbonic anhydrase I. It is of interest, however, that Mushak and Coleman (39) have found that label VIII is immobilized at the active site of both bovine carbonic anhydrase II and human carbonic anhydrase II but is fairly mobile when bound to human carbonic anhydrase I and monkey carbonic anhydrase I. A molecular model of label VIII shows quite clearly that the distance between the sulfonamide nitrogen atom and the piperi-

dine ring is about the same as it is in label VII (Table 2 and Fig. 6). It seems unlikely that molecular size can account for our observations, since the piperidine ring of labels VII and VIII is more bulky than the pyrrolidine ring of label II, and yet these labels exhibit higher mobility at the active site of type II isozymes. Molecular models do indicate, however, that in the fully extended conformation the $x$ axis of the nitroxide group in label VII is roughly parallel to the long axis of the molecule, while in label II the $x$ axis is at an angle of about 30° to the long axis. Thus it would seem possible that steric interactions between the pyrrolidine ring of label II and the active site of human carbonic anhydrase I may result in the greater immobilization of this label. Whatever the molecular property of label VII that results in these differences, it is still obvious that this label has the capacity to distinguish between type I and type II carbonic anhydrases. This finding is of interest, because Maren and co-workers have recently pointed out (40) that the carbonate hydro-lyase activity of human carbonic anhydrase I would be almost completely inhibited by the normal physiological concentration of chloride ion present in red cells. They have suggested that perhaps some substrate other than either carbon dioxide or bicarbonate may be metabolized by the type I carbonic anhydrases. Thus it would seem possible that the differences between the active sites of type I and II carbonic anhydrases which are reported here may reflect a difference in the substrate specificities of the two isozymes.

While the shorter chain length pyrrolidine analogues (I and II) cannot distinguish between the type I and type II isozymes, it does seem that the longer chain labels (III–V) are capable of differentiating between them. Thus, when two isozymes from the same species are compared, e.g., human or rhesus monkey, labels III–V exhibit greater mobility at the active site of type I isozymes. A similar result was obtained with horse carbonic anhydrases I and II (27). It therefore appears that type II isozymes may be somewhat shallower than type I. This suggestion finds additional support from the observation that for most of the type II isozymes there is little difference between the mobilities of the pyrrolidine rings of labels IV and V (Fig. 3).

The finding that when spin label II binds to rabbit carbonic anhydrase II there are two populations of bound spin labels is of some interest. Similar results have also been obtained with label III bound to human carbonic anhydrase I (26), bovine carbonic anhydrase II (25), and horse carbonic anhydrase I (27). There are two possible explanations for this observation. The first is that there are two different solution conformations of the enzyme, in which the active site differs somewhat in its size and shape. This would appear unlikely. A more reasonable explanation is that the two diastereoisomeric forms of spin label II interact differently at the active site of these isozymes. A similar explanation has been proposed by Berliner and Wong (41) in their studies of *p*-toluenesulfonyl spin labels covalently bound to trypsin and chymotrypsin.

These studies have shown that spin-labeled analogues of sulfonamides are useful probes for the active site of erythrocyte mammalian carbonic anhydrases. A comparison of the results obtained by the spin-labeling technique with the X-ray crystallographic data available for human carbonic anhydrases I and II appears to validate the "molecular dipstick" technique for probing the depth of drug-combining sites. While it is not possible to account for the observed differences between the ESR spectra of spin labels II and VII bound to the active sites of human carbonic anhydrases I and II, it nevertheless appears that spin label VII can differentiate between type I and type II isozymes.

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