Drug–Biomolecule Interactions: Topographical Study of Active Site of Erythrocyte Carbonic Anhydrase Using Spin-Labeled Sulfanilamide Drugs

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Abstract □ The topography of the active sites of human erythrocyte carbonic anhydrases B and C and bovine erythrocyte carbonic anhydrase B was studied using a series of spin-labeled sulfanilamide analogs. Results show that the active site of human carbonic anhydrase C is a narrow cleft approximately 14 Å in depth. This observation is in good agreement with previously published X-ray diffraction data. While the active sites of human carbonic anhydrase B and bovine carbonic anhydrase B have the same general shape as the active site of human carbonic anhydrase C, they are slightly deeper.

Keyphrases □ Erythrocyte carbonic anhydrase—active site, topographical study, spin-labeled sulfanilamide drugs □ Carbonic anhydrase, erythrocyte—active site, topographical study, spin-labeled sulfanilamide drugs □ Sulfanilamide drugs, spin labeled—topographical study of active site of erythrocyte carbonic anhydrase □ Spin-labeled drugs—sulfanilamide, topographical study of active site of erythrocyte carbonic anhydrase □ Drug–biomolecule interactions—topographical study of active site of erythrocyte carbonic anhydrase using spin-labeled sulfanilamide drugs □ Interactions—drugs with biomolecules, symposium

Spin labels are stable free radicals that can be used as probes or reporter groups for biological macromolecules such as proteins, nucleic acids, and membranes. The first free radical to be used as a spin label was the chlorpromazine cation radical. Ohnishi and McConnell (1) studied the interaction of this spin label with DNA and found that it was bound with its aromatic plane nearly perpendicular to the helix axis of the nucleic acids. Since the chlorpromazine radical was only moderately stable in aqueous solutions, other, less reactive, spin labels were sought. The synthesis of di-*tert*-butylnitroxide (2), a free radical that was very stable in aqueous systems over wide temperature and pH ranges, had been described previously. The unique chemical and physical properties of the nitroxide group made it an ideal spin label, and various molecules containing this free radical have now been synthesized (3).

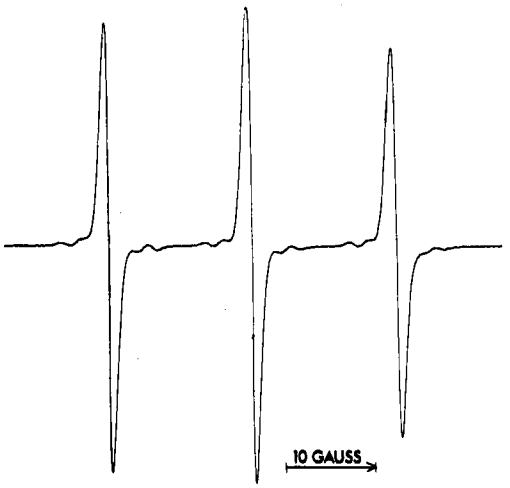


Figure 1—*Electron spin resonance spectrum of II* (2×10^{-5} M) *in 0.1* M *sodium phosphate buffer.*

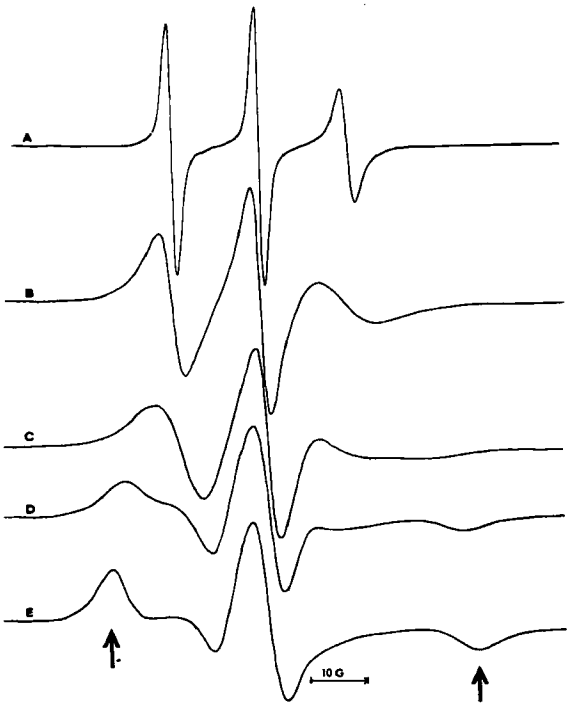
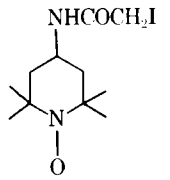


Figure 2—*Electron spin resonance spectrum of III* (1×10^{-4} M) *dissolved in glycerol. Key: A, 60°; B, 30°; C, 20°; D, 10°; and E, 0°. The two arrows indicate the positions of the low (left) and high (right) field peaks which are characteristic of a highly immobilized nitroxide radical.*

DISCUSSION

When a nitroxide spin label is dissolved in a solvent of low viscosity, such as aqueous buffer, its electron spin resonance spectrum consists of three sharp lines of nearly equal height (Fig. 1). This spectrum may be characterized by three parameters: *(a)* the position of the center line in the magnetic field (this is governed by a constant, g_0, termed the g factor); *(b)* the distance between adjacent lines, A_0, measured in gauss (G); and *(c)* the individual linewidths measured in gauss. When the environment of the nitroxide group is altered, then changes may occur in one or more of these parameters (3).

For example, di-*tert*-butylnitroxide in water yields $A_0 = 16.7$ G and $g_0 = 2.0056$, whereas di-*tert*-butylnitroxide in hexane is characterized by $A_0 = 14.8$ and $g_0 = 2.0061$. When a glycerol solution of a nitroxide spin label is cooled, the electron spin resonance spectrum of the free radical becomes broad and asymmetric (Fig. 2).



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The limiting line shape (Fig. 2E), which is known as the rigid glass, polycrystalline, or powder spectrum of the nitroxide radical, is characterized by the appearance of distinct low and high field peaks separated by approximately 64 G (Fig. 2E).

The rigid glass spectrum is observed when the spin label is randomly oriented with respect to the laboratory magnetic field and the molecular motion of the nitroxide group is slow on the electron spin resonance time scale, *i.e.*, $\tau_c \gg 10^{-8}$ sec, where τ_c is the rotational correlation time of the spin label. Spectra approaching the rigid glass limit have been observed when nitroxide spin labels were tightly bound to macromolecules such as proteins and membranes (3).

Nitroxide spin labels can be employed in two different ways to study drug–biomolecule interactions. First, they can be attached (covalently or noncovalently) to the macromolecule of interest and function as reporter groups. This approach was used by Holmes and Piette (4) to study the interaction of chlorpromazine with erythrocyte ghost membranes. When these workers reacted an iodoacetamide spin label (I) covalently with the erythrocyte membrane, the electron spin resonance spectrum of the labeled membranes indicated that the nitroxide group had a high degree of freedom.

It would, therefore, appear that the protein sulfhydryl groups labeled by this reagent were on the outside of the membrane. When chlorpromazine was added to the spin-labeled membranes, a highly immobilized component appeared in the electron spin resonance spectrum of the nitroxide group. Holmes and Piette (4) suggested that chlorpromazine induced a conformational change in erythrocyte membranes so that some spin labels were moved into the interior. Since the highly immobilized component disappeared on removal of chlorpromazine, the conformational change was apparently reversible.

Hubbell *et al.* (5) used a similar approach to study the interaction of local anesthetic drugs, such as benzyl alcohol and lidocaine, with erythrocyte membranes. The spin labels employed included nitroxide analogs of methyl stearate and 17β-hydroxy-5α-androstane, which were incorporated noncovalently into red cell membranes. The steroid spin label rotated preferentially about its long axis, which was oriented perpendicular to the membrane surface. The addition of benzyl alcohol produced a progressive sharpening of the electron spin resonance spectrum of the spin label, which suggested that the environment of the free radical was becoming more fluid.

However, at high (lytic) concentrations of benzyl alcohol, a new spectral component appeared, which corresponded to a fraction of spin labels that were in a highly immobilized environment. This same component was also observed when the androstane spin label bound to protein was isolated from the erythrocyte membrane. Thus, the highly immobilized component that appeared in the lytic concentration range of benzyl alcohol was attributed to new protein binding sites for the spin label. These results agreed well with previous NMR studies of benzyl alcohol binding of erythrocyte membranes (6).

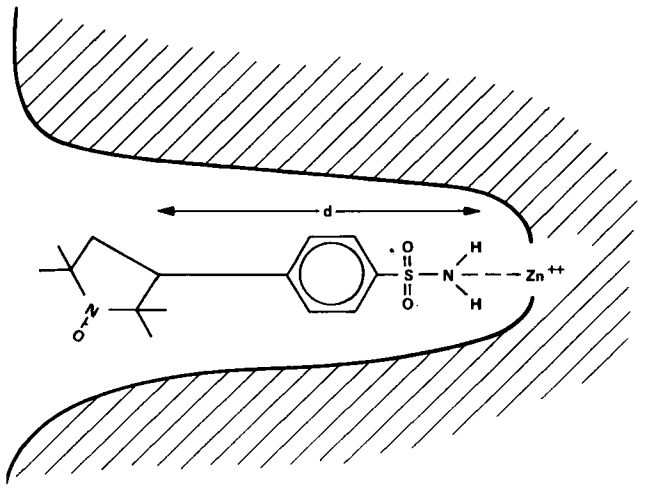


Figure 3—*Active site of human carbonic anhydrase C, showing the binding of a spin-labeled sulfanilamide analog.*

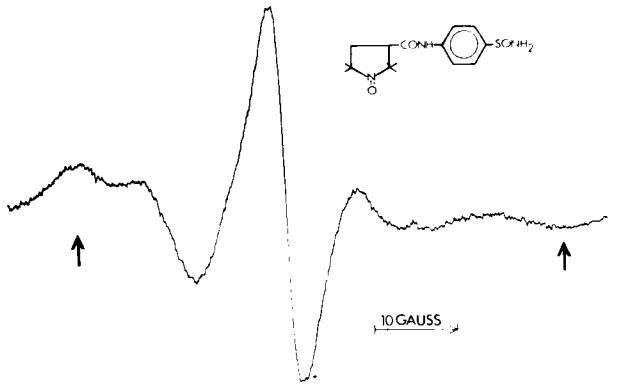


Figure 4—*Electron spin resonance spectrum of II (2×10^{-5} M) in the presence of human erythrocyte carbonic anhydrase C (1×10^{-4} M) and 0.1 M sodium phosphate buffer. The arrows indicate the positions of the low (left) and high (right) field peaks which are characteristic of a highly immobilized nitroxide radical* (cf., *Fig. 2E*).

The second way in which spin labels can be used to study drug interactions with biological systems is to incorporate the nitroxide group into the drug molecule. This approach has been used to probe the active site of erythrocyte carbonic anhydrase, a zinc-containing metalloenzyme which is inhibited by aromatic sulfonamide drugs with the general formula $ArSO_2NH_2$ (where Ar is homoaromatic or heteroaromatic).

X-ray diffraction studies of human carbonic anhydrase C have shown (7, 8) that the active site of this enzyme is a deep crevice, at the bottom of which is a single zinc atom (Fig. 3). When 3-acetoxymercuri-4-aminobenzenesulfonamide combined with human carbonic anhydrase C, the aromatic group of the inhibitor occupied a narrow cleft in the active site while the sulfonamide group was coordinated directly to the zinc through its nitrogen atom or one of the oxygen atoms (7). The topography of the active site of human carbonic anhydrase C and two other erythrocyte carbonic anhydrases was studied using a series of spin-labeled analogs of the inhibitor sulfanilamide (II–VI) in which the length of the chain separating the aromatic and pyrrolidine rings was progressively increased (Fig. 3 and Table I).

Before an electron spin resonance study of the interaction of the sulfonamide spin labels with human carbonic anhydrase C was attempted, it was necessary to establish that these compounds did indeed bind to the active site by measuring their ability to inhibit the enzymatic activity of the enzyme. Although all spin-labeled

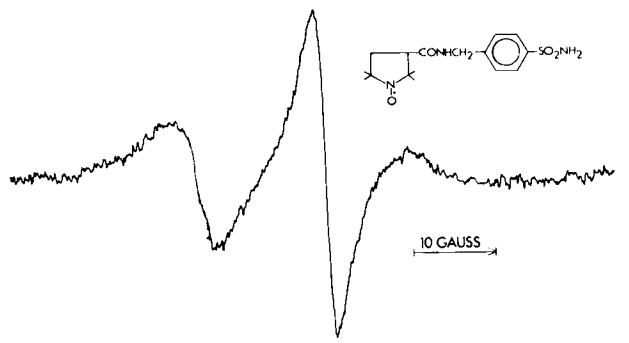
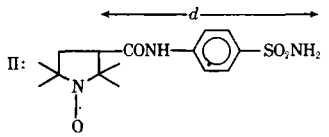
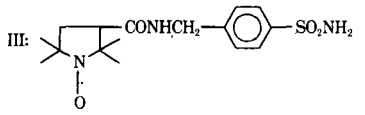
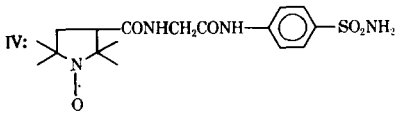
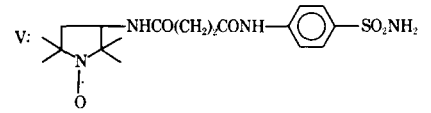
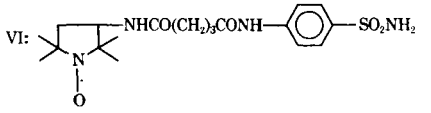


Figure 5—*Electron spin resonance spectrum of III (2×10^{-5} M) in the presence of human erythrocyte carbonic anhydrase C (1×10^{-4} M) and 0.1 M sodium phosphate buffer.*

Table I—Inhibition of the Esterase Activity of Human Erythrocyte Carbonic Anhydrases B and C by Spin-Labeled Sulfonamides

Spin Label	K_I, $M \times 10^6$			d^a, Å
	Bovine Carbonic Anhydrase B	Human Carbonic Anhydrase B	Human Carbonic Anhydrase C	
II: 	0.02	0.33	0.08	10.0
III: 	0.42	0.26	1.89	10.8
IV: 	2.71	2.78	0.58	13.6
V: 	0.78	3.94	1.11	14.5
VI: 	—	—	—	15.6

[a] Determined with Pauling–Corey–Koltun models for the fully extended conformation of each sulfonamide spin label.

sulfonamides did inhibit the ability of human carbonic anhydrase C to hydrolyze *p*-nitrophenylacetate (9), the kinetics of the interaction were noncompetitive in all cases (Table I). While this suggested that the sulfonamides did not bind to the active site of the enzyme, similar noncompetitive kinetics had already been reported for the inhibition of the esterase activity of erythrocyte carbonic anhydrase by other sulfonamides such as acetazolamide (9). Yet it is known, from X-ray diffraction studies, that these drugs bind to the active site of human carbonic anhydrase C (7, 8). Kernohan (10) suggested that the apparent noncompetitive nature of the kinetics is due to a relatively slow rate of dissociation of the sulfonamide from the active site of a carbonic anhydrase.

The electron spin resonance spectrum of II bound to human carbonic anhydrase C was broad and asymmetric, with a splitting of 59 G between the low and high field peaks (Fig. 4). Since this spectrum resembled the rigid glass spectrum of the nitroxide group (Fig. 2E), there was little doubt that the pyrrolidine ring of II was highly immobilized when this inhibitor was bound to human carbonic anhydrase C. When a single methylene group was inserted between the benzene ring and the aromatic amino group of II to give III, the nitroxide radical showed some increase in mobility at the active site of human carbonic anhydrase C (Fig. 5).

In the complex between IV and human carbonic anhydrase C, the nitroxide group exhibited considerable freedom of movement at the active site of the enzyme (Fig. 6). A further increase in the distance between the aromatic and pyrrolidine rings, to give V, did not greatly change the electron spin resonance spectrum of the spin label (Fig. 7). In their spin-label study of hapten binding to rabbit immunoglobulins, Hsia and Piette (11) obtained electron spin resonance spectra that were similar to these shown in Figs. 6 and 7. They suggested that such spectra were characteristic of spin-labeled ligands bound to rather narrow combining sites in such a way that their nitroxide groups were just outside the sites. The mobilities of the nitroxide groups shown in Figs. 5–7 can be expressed in terms of their respective rotational correlation times (12). The rotational correlation times of the nitroxide groups of III–VII reached a minimum with IV, which did not change when the chain joining the aromatic and pyrrolidine rings was further extended (Fig. 8).

Further studies showed that the pyrrolidine ring of II is also highly immobilized when this inhibitor binds to the active site of human erythrocyte carbonic anhydrase B and bovine erythrocyte carbonic anhydrase B (14). However, when the chain length separating the aromatic and pyrrolidine rings was increased, V was the first spin label of the series in which the nitroxide group showed high mobility at the active site of human carbonic anhydrase B. While similar results were obtained for bovine carbonic anhydrase B, it was also found that the nitroxide group of VI had less mobility at the active site of this enzyme than did the nitroxide group of V (Fig. 8). This finding suggested that VI may have been sufficiently flexible as to allow the pyrrolidine ring to turn back and interact with either the active site of bovine carbonic anhydrase B or an accessory binding site on the surface of the enzyme (14).

The electron spin resonance spectrum of II bound to human carbonic anhydrase C indicated quite clearly that the nitroxide group of the inhibitor was highly immobilized at the active site of the enzyme. This observation suggests that the active site of human carbonic anhydrase C is a narrow cleft and is in agreement with X-ray diffraction data of Bergstén *et al.* (8). Sulfonamide IV was the first

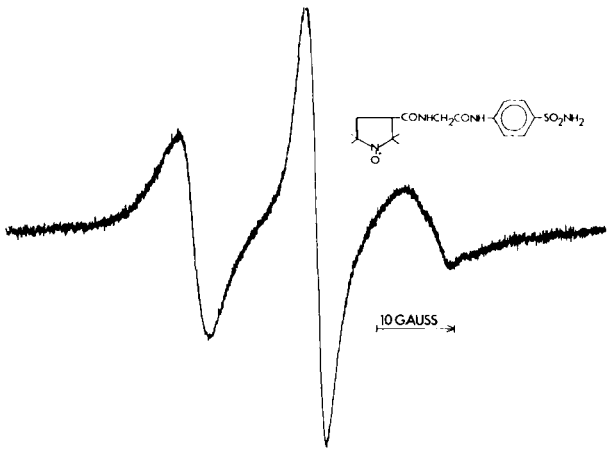


Figure 6—*Electron spin resonance spectrum of IV* (2×10^{-5} M) *in the presence of human erythrocyte carbonic anhydrase C* (1×10^{-4} M) *and 0.1 M sodium phosphate buffer.*

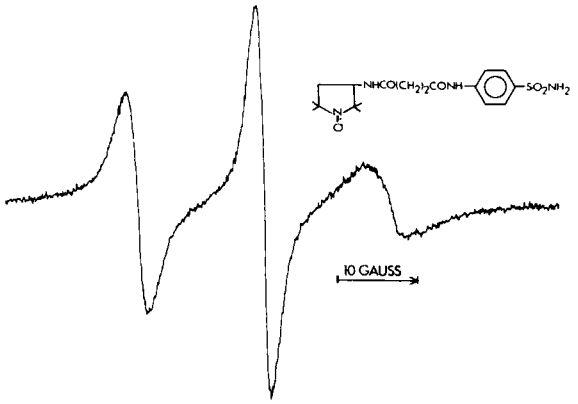


Figure 7—*Electron spin resonance spectrum of V (2×10^{-5} M) in the presence of human erythrocyte carbonic anhydrase C (1×10^{-4} M) and 0.1 M sodium phosphate buffer.*

of the spin labels in which the pyrrolidine ring appeared to be outside the active site of human carbonic anhydrase C.

The distance, *d*, between the nitrogen atom of the sulfanilamide group and the 3-position of the pyrrolidine ring can be estimated from Pauling–Corey–Koltun models to be 13.8 Å for the fully extended conformation of IV (Table I). This would set an upper limit of 14 Å for the depth of the active site of human carbonic anhydrase C. This value is in good agreement with an estimate of 15 Å obtained from X-ray diffraction studies (8). Although the active sites of human carbonic anhydrase B and bovine carbonic anhydrase B appear to have the same shape as human carbonic anhydrase C, they probably are slightly deeper.

These studies have shown that spin-labeled sulfonamides can be used to study the topography of the active site of erythrocyte carbonic anhydrase. It is hoped that spin-labeled analogs of other drug molecules, such as acetylcholine and the barbiturates (15), may make it possible to investigate their interactions with receptor biomolecules. The spin label technique also permits the rapid measurement of low concentrations of sulfonamide drugs present in biological fluids (16). A simple assay procedure for the estimation of carbonic anhydrase levels in red cell hemolysates has also been developed (15) and employs a spin-labeled sulfonamide. There now seems to be little doubt that electron spin resonance and the spin-labeling technique will not only be important tools for studying drug–biomolecule interactions at a molecular level but also may provide a versatile and sensitive assay procedure for drugs, enzymes, and other binding proteins.

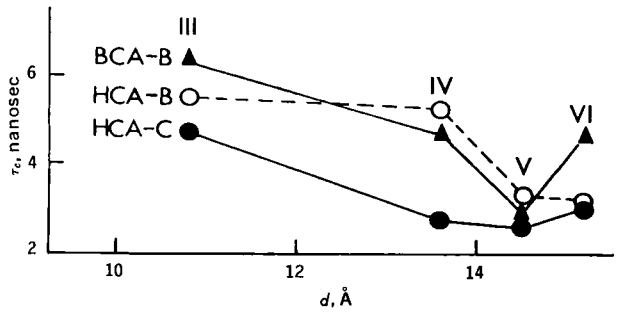


Figure 8—*Rotational correlation times, τ_c, of III–VI as a function of the distance, d, between the sulfonamide nitrogen atom and the point of attachment of the pyrrolidine ring (Fig. 3 and Table I).*

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