Combination of Bovine Carbonic Anhydrase with a Fluorescent Sulfonamide

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SUMMARY

Bovine erythrocyte carbonic anhydrase forms a highly fluorescent complex with 5-dimethylaminonaphthalene-1-sulfonamide (DNSA). The binding, studied either by enhancement of ligand fluorescence or by the quenching of protein ultraviolet fluorescence, shows that only 1 mole of DNSA is bound per mole of protein; the dissociation constant at pH 7.4 is \(2.5 \times 10^{-7}\) M. The fluorescence of free DNSA in water has peak emission at 580 nm and a quantum yield of only 0.055, but bound DNSA has an emission maximum at 468 nm and a yield of 0.84. Arguments are presented to explain the large emission blue shift on the basis that the binding site is extremely hydrophobic and that the \(-\text{SO}_2\text{NH}_2\) group of the ligand loses a proton upon binding to the enzyme. The binding appears specifically to involve the sulfonamide site known to exist in carbonic anhydrase; several other "fluorescent probe" compounds showed no evidence of binding to the enzyme.

Calculation of the energy transfer efficiency gave the surprising result that 85% of the photons absorbed by the 7 tryptophan residues are transferred to the single bound DNSA molecule. The transfer efficiency is much higher than hitherto observed for a protein having only one 5-dimethylaminonaphthalene-1-sulfonyl group. Although the diameter of the protein is roughly 51 Å, the bound DNSA group is probably within the critical transfer distance \(R_0\) (≈ 21.3 Å) of all the tryptophans. The effective average distance between DNSA and tryptophan was found to be 16 Å. The fluorescence properties of the complex were quite different from those of a conjugate prepared by reaction of 5-dimethylaminonaphthalene-1-sulfonyl chloride with carbonic anhydrase. Various considerations lead to the conclusion that the sulfonamide-binding site and the tryptophan residues are in the interior of the protein.

The tryptophan fluorescence of the protein was 73% quenched by the binding of 1 DNSA molecule. Although large, this degree of quenching was less than the over-all efficiency of energy transfer of photons absorbed by the protein. This result indicates that the fluorescence efficiencies of the 7 tryptophans are different, and that DNSA is bound in such a way that energy transfer occurs with greater probability from those tryptophan residues which are relatively less fluorescent.

DNSA inhibits the esterase activity of carbonic anhydrase as tested with the substrate, \(p\)-nitrophenyl acetate. Direct measurements of fluorescence decay times permitted calculation of the rotational relaxation time of carbonic anhydrase from depolarization of fluorescence data. The value of about 30 nsec for the relaxation time is consistent with a low degree of molecular asymmetry.

In 1940, Mann and Keilii (1) reported the discovery that sulfonamides inhibit carbonic anhydrase. The reason for the highly specific and potent inhibition of this enzyme by this class of compounds is still not clear, although a number of facts bearing on the question is known. All sulfonamides which inhibit animal carbonic anhydrases have the \(-\text{SO}_2\text{NH}_2\) group attached directly to an aromatic nucleus, which is either homocyclic or heterocyclic (2). The effectiveness of the inhibitor seems to be related to the ease with which a proton can disassociate from the sulfonamide group. There is evidence that such an ionization occurs before the inhibitor binds to the enzyme (3, 4). Inhibitory sulfonamides appear to be bound to the enzyme at a site which involves the metal ion at the active center (5, 6), and the sulfonamide-binding site may be identical with, or may overlap, the CO₂-binding site (7). However, sulfonamides do not form strong coordination bonds with transition metal ions such as Zn²⁺, and an interaction of the aromatic nucleus of the inhibitor with the protein must presumably make a large contribution to the observed stability of the complexes which are formed.

In this paper, we present some evidence for a hydrophobic binding site in bovine erythrocyte carbonic anhydrase B based on the properties of a highly fluorescent complex of the enzyme containing the sulfonamide, DNSA. The results of fluorometric

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treatments show that only 1 DNSA molecule is bound by carbonic anhydrase. Emission spectral measurements show that the DNSA must be bound to a region of very low dielectric constant, and energy transfer calculations indicate that the sulfonamide may be in close association with some, if not most, of the tryptophan residues. In short, DNSA is a "fluorescent probe" of the sulfonamide-binding site of carbonic anhydrase. Several fluorescent dyes have been used as probes of hydrophobic binding sites (8-10). Typical of this class of dyes is 8-anilinonaphthalene-1-sulfonic acid, which is virtually nonfluorescent when free in aqueous solution but is reported to have a quantum yield of 0.75 when bound to bovine serum albumin (8). The spectra and quantum yields of such dyes are sensitive to conformational changes, which may affect the properties of the binding site (11-13). Recently, it was reported (14) that the DNS-derivatives of amino acids (so-called dansylamino acids) have properties similar to those of ANS and can be used as fluorescent probes; thus, it is not altogether surprising that DNSA can also fulfill this function. However, the binding of DNSA to carbonic anhydrase appears to involve those factors which render the inhibition by sulfonamides so specific and effective, and provides information not otherwise obtainable concerning interaction with this class of ligands. Neither ANS nor the DNS-amino acids, on the other hand, appear to bind to this enzyme.

**EXPERIMENTAL PROCEDURE**

**Reagents**—Electrophoretically pure bovine carbonic anhydrase B was used in all experiments, and was the same sample used in a previous study (4). Enzyme concentrations were estimated spectrophotometrically, with ε_{250} = 5.7 \times 10^4 M^{-1} cm^{-1} (15). Bovine serum albumin was a crystalline sample obtained from Armour (Lot AA9005) and defatted by charcoal treatment (16). DNSA was prepared according to Weber (17) and recrystallized from ethanol. Stock solutions containing 10^{-3} M DNSA were made by dissolving a weighed amount of the dried sulfonamide in 10^{-3} M HCl; these solutions were stable for at least 1 week. The extinction coefficient of DNSA in 60\% (v/v) ethanol and the absorption spectra at various pH values matched reported values (17, 18). Purity of DNSA was also confirmed by thin layer chromatography in several solvent systems (19). ANS (Baker) was recrystallized as the magnesium salt (8). DNS-derivatives of glycine, l-proline, l-glutamic acid, and \alpha-tryptophan were purchased from British Drug Houses, and their purity was established by thin layer chromatography (19). Ethoxylamide (6-ethoxybenzothiazole-2-sulfonamide) was a gift from Upjohn and was used without further purification. DNS-chloride was obtained from Baker and recrystallized from acetone before use.

**Esterase Activity of Carbonic Anhydrase**—The procedure of Armstrong, Myers, Verpoorte, and Edsall (20) was followed, except that the buffer was 0.02 M \text{HzSO}_4, and as-

**Optical Measurements**—Absorbance was measured in Beckman DU and Cary model 11 spectrophotometers. Fluorescence intensities and spectra were obtained with Aminco-Bowman spectrophotofluorometers. Cells of 1-cm path length were used for fluorometric titrations, but a microcell having internal cross-sectional dimensions of 0.20 x 0.29 cm was used for spectral measurements. Detailed description of the instrumentation, its use, the methods of calibration to give corrected excitation and emission spectra, and the calibration curves have been published (21, 22). Fluorescence quantum yields were obtained by comparing the area under the corrected emission spectrum with that of a standard with a known quantum yield; the procedure was the same as that used in a previous study (23). Fluorescence polarization, P, was measured with the Aminco-Bowman apparatus with linearly polarized excitation (27). We calculated P from the relation (28)

\[ P = \frac{I_{\text{VV}} - G_{\text{VV}}}{I_{\text{VV}} + G_{\text{VV}}} \]

where I is the observed fluorescence intensity and the first and second subscripts refer to the orientation of the polarizer and analyzer, respectively, and G is a correction factor for polarization introduced by the emission monochromator grating and is given by \[ G = I_{\text{VV}}/I_{\text{HH}}. \]

Fluorescence decay time, \( \tau \), was measured with a TRW Instruments nanosecond flash apparatus, which was modified as previously described (29).

Unless otherwise specified, all experiments were conducted in 0.02 M phosphate buffer, pH 7.4, at 24 \pm 1°C.

**RESULTS**

**Binding of DNSA by Carbonic Anhydrase**—When DNSA was added to a solution of enzyme, the protein fluorescence excited at 280 m\( \mu \text{m} \) was quenched. The stoichiometry of the interaction of carbonic anhydrase with DNSA was studied by titration of a 10^{-4} M solution of protein with the sulfonamide, and the results are shown in Fig. 1. The quenching curve indicates that only 1 mole of ligand is bound per mole of protein. Since the extinction coefficients of the sulfonamide and the protein are known with some certainty (15, 18), the quenching curve can be used to calculate the molecular weight of the protein. With a solution of a weighed amount of carbonic anhydrase, a value of 29,500 was obtained, in good agreement with the value obtained by Nyman and Lindskog (15) from amino acid composition.

The binding of DNSA by carbonic anhydrase is accompanied by enhancement of ligand fluorescence, which again is consistent with the binding of 1 mole of sulfonamide (Fig. 1). Although it could be argued that additional DNSA might be bound without...
stoichiometry but also to obtain the dissociation constant,

$$K_D = \frac{[EN]}{[E][N]}$$

where $[EN]$ is the concentration of enzyme-ligand complex, $[E]$ is the concentration of enzyme, and $[N]$ is the concentration of ligand.

Fig. 1. Fluorometric titration of carbonic anhydrase at high concentration $(8.2 \times 10^{-6} \text{ M})$ with DNSA. The intensity of tryptophan fluorescence (solid line) was followed by excitation at 280 nm, with the emission monochromator set at 336 nm; the corresponding wave lengths for ligand fluorescence (dotted line) were 320 and 470 nm. The emission of free DNSA at 470 nm was negligible. Band widths of excitation and emission were 12 nm.

Fluorescence enhancement, this possibility is quite unlikely since one would then expect further quenching of protein fluorescence. The binding data of Fig. 1 could be used not only to show the binding stoichiometry but also to obtain the dissociation constant, $K_D$. However, $K_D$ was obtained with greater precision by titrations at low enzyme concentration, as shown in Fig. 2. $K_D$ was found to be 2.4 $(\pm 0.1) \times 10^{-7} \text{ M}$. The experiment of Fig. 2 illustrates a binding stoichiometry 1:1 complex with carbonic anhydrase, with the emission wave lengths shown.

The protein concentration was $2.1 \times 10^{-7} \text{ M}$. The fraction of enzyme bound was assumed equal to the fraction of the maximum degree of DNSA fluorescence enhancement or tryptophan fluorescence quenching as determined in experiments similar to that of Fig. 1.

However, $K_D$ was obtained with greater precision by titrations at low enzyme concentration, as shown in Fig. 2. $K_D$ was found to be 2.4 $(\pm 0.1) \times 10^{-7} \text{ M}$. The experiment of Fig. 2 illustrates a point which will be expanded on in another communication; namely, that fluorescence quenching is a very sensitive method of obtaining the binding constants of certain aromatic sulfonamides by carbonic anhydrase and permits titrations of solutions containing $10^{-7}$ to $10^{-4} \text{ M}$ protein.

The 1:1 binding stoichiometry was also confirmed by another method. Ethoxzolamide, a highly potent inhibitor, has been found by fluorescence quenching data to form a 1:1 complex with carbonic anhydrase with a $K_D$ of $2.5 \times 10^{-10} \text{ M}$. Ethoxzolamide has negligible visible fluorescence; when it was added to solutions containing both carbonic anhydrase and DNSA, the enhancement of DNSA emission was blocked when equivalent amounts of enzyme and ethoxzolamide were present. The results of such a competition experiment are given in Fig. 3. Since ethoxzolamide is used clinically as a carbonic anhydrase inhibitor, the data provide evidence that the DNSA site is the binding site for such sulfonamide inhibitors.

**Energy Transfer from Protein to DNSA**—The quenching of carbonic anhydrase fluorescence by DNSA, like that observed in other protein interactions (30–32), is due to energy transfer from excited state tryptophanyl residues to bound ligand. A general prerequisite for energy transfer to occur by the Förster mechanism (33), involving induced dipole resonance, is that the emission band of the donor overlap the absorption band of the acceptor. Fig. 4 shows that this prerequisite is met in the system CA(DNSA), since the protein fluorescence band ($\lambda_{max}$ 336 nm) strongly overlaps the DNSA absorption band. The probability of energy transfer between two molecules can be expressed in terms of the critical transfer distance, $R_0$, for which resonance transfer is 50% complete by means of the equation (34, 35)

$$R_0 = \frac{6}{1.66 \times 10^{-6} \times n^2 \times \lambda^3}$$

where $\lambda$ is the donor fluorescence decay time, $n_0$ the mean of the peak positions (in wave numbers) of the donor emission and lowest energy absorption bands, $J_\alpha$ the overlap integral, and $n$ the refractive index. Stryer (36) has applied the equation to proteins bearing DNSA groups and has obtained $R_0 = 21 \text{ A}$ for transfer from tryptophan to dye.

We have recalculated $R_0$ from Equation 1 from the spectral data of Fig. 4 for CA(DNSA), since it cannot be assumed that all DNS-protein systems have the same spectra. To obtain $R_0$ we redrew the data of Fig. 4 in terms of the wave number scale, taking care to multiply the corrected emission spectrum of carbonic anhydrase by $n^2$ according to Lippert et al. (37) in order to display the curve as relative quanta (d$I$/d$\lambda$) with respect to $\lambda$. By assuming that $n = 1.6$, and by using $\tau = 2.6$ nsec (directly measured), we obtained $J_\alpha = 0.61 \times 10^5 \text{ cm}^2 \text{ mole}^{-1}$, $R_0 = 32.8 \times 10^4 \text{ cm}^{-1}$, and $R_0 = 21.3 \text{ A}$. The good correspondence of $R_0$ with that calculated by Stryer (36) for other proteins shows that the high quenching efficiency of DNSA on carbonic anhydrase is not due to any unusual spectral features such as an increase in oscillator strength or overlap integral; rather, the observations

![Fig. 1. Fluorometric titration of carbonic anhydrase at high concentration (8.2 x 10^-6 M) with DNSA. The intensity of tryptophan fluorescence (solid line) was followed by excitation at 280 nm, with the emission monochromator set at 336 nm; the corresponding wave lengths for ligand fluorescence (dotted line) were 320 and 470 nm. The emission of free DNSA at 470 nm was negligible. Band widths of excitation and emission were 12 nm.](http://www.jbc.org/)

![Fig. 2. Binding of DNSA to carbonic anhydrase in dilute solution, measured by quenching of protein fluorescence and enhancement of ligand fluorescence, with the emission wave lengths shown. The protein concentration was 2.1 x 10^-7 M. The fraction of enzyme bound was assumed equal to the fraction of the maximum degree of DNSA fluorescence enhancement or tryptophan fluorescence quenching as determined in experiments similar to that of Fig. 1.](http://www.jbc.org/)

![Fig. 3. Effect of ethoxzolamide on the visible fluorescence of CA(DNSA). Small aliquots of 10^-3 M ethoxzolamide were added to a solution containing 5.6 x 10^-3 M carbonic anhydrase and 3 x 10^-6 M DNSA. Fluorescence was observed at 470 nm; excitation, at 320 nm.](http://www.jbc.org/)
on quenching must be explained by particularly favorable spatial or geometrical factors, or both.

We recognize, however, that the transfer distance, $R_0$, calculated in the above manner is an approximation, since several assumptions must be made in order to use Equation 1 for protein-ligand systems. For instance, it is assumed that the orientations of the tryptophans of the protein are random with respect to the DNSA, that the various tryptophans all have the same emission spectra and lifetime, and that the refractive index in the protein matrix is 1.6. Nonetheless, because the transfer distance $R_0$ is proportional to the sixth root of the ratio $J_0/n^2$, a 100% error in that quantity would result in only a 10% error in $R_0$.

The binding of only 1 mole of DNSA to carbonic anhydrase results in 73% quenching of protein fluorescence (Fig. 1). Because it is known that bovine carbonic anhydrase has 7 tryptophan residues (15), it seemed possible that quenching might involve certain residues more than others and therefore result in a change in the ultraviolet emission spectrum. However, with 290 m$\mu$ excitation, the emission spectra of carbonic anhydrase and of CA(DNSA) had the same shape and position. Thus, we cannot from this result distinguish between the following two possibilities: (a) the emission from the fluorescent tryptophans is 73% quenched by DNSA, or (b) the fluorescence of certain tryptophans is 100% quenched while that of other residues is quenched to a lesser degree, yet the resultant protein emission spectrum is unchanged since all the emitting residues have the same spectra.

More information on possible differences between the tryptophan residues is, however, obtained from the following considerations. Although the degree of quenching is related to the energy transfer process, the true efficiency of tryptophan-DNSA energy transfer cannot be calculated from quenching data alone, for the following reason. The quantum yield of tryptophan fluorescence in carbonic anhydrase is only 0.11 (Table I); thus 89% of the absorbed photons are lost by radiationless pathways. Since the quenching data concern only those 11% of the photons which are ordinarily emitted, one must turn to a comparison of the absorption and corrected excitation spectra of CA(DNSA) to determine the percentage of photons transferred to DNSA. Such spectra are shown in Fig. 4. The absorption spectrum for the solution containing DNSA and carbonic anhydrase is essentially that of CA(DNSA), since, under the conditions used, only 9% of the enzyme present was not in complex with the sulfonamide. Suitable corrections in the quantum yield determinations were applied for this amount of free enzyme. The excitation spectrum of CA(DNSA) is of interest because of the intense 280 m$\mu$ peak corresponding to the long wave length protein absorption band, whereas the spectrum of DNSA itself has a minimum in

It should be noted that the fluorescence quantum yield of protein fluorescence was obtained with the quinine standard rather than with a tryptophan standard, which is often used. The quantum yield of tryptophan in water has been reported to be 0.20 (38), but experiments with various spectrofluorometers and with quinine as a standard have shown tryptophan to have a quantum yield of 0.13 in water (R. F. Chen and R. Perlman, to be published). The quantum yield of carbonic anhydrase is thus only slightly less than that of tryptophan which is free in aqueous solution.
this region. Above 300 \text{ nm}, the absorption and excitation spectra coincide and the quantum yield is 0.84. In the region where light is absorbed by the protein, the fluorescence quantum yield is lower. After correcting for the amount of light directly absorbed by bound DNSA, we found that the quantum yields of the visible fluorescence excited by photons absorbed at 280 \text{ nm} and 290 \text{ nm} by the protein were 0.68 and 0.71, respectively. The difference in yields at these two wave lengths merely reflects the lower degree of energy transfer from tyrosine residues (36), which contribute to absorption at 280 \text{ nm} much more than at 290 \text{ nm}. From the result at 290 \text{ nm}, it is evident that the quanta absorbed by tryptophan residues are transferred to DNSA with a yield of 85\% (0.71/0.84 \times 100). Also, since the quantum yield of tryptophan fluorescence in the complex if 0.04 (quantum yield data are summarized in Table I), we can account for 80\% of the photons absorbed by tryptophan. The remaining 11\% presumably suffer radiationless internal conversion directly from excited state tryptophans. The high degree of energy transfer means that DNSA very effectively scavenges those photons which, in the free enzyme, would have been lost by radiationless modes. For this reason, although the lifetime of the free enzyme fluorescence is found to be only 2.6 nsec, the energy transfer process must be considerably faster than even that. The rapidity of energy transfer was confirmed by measuring the fluorescence decay time of CA(DNSA) by excitation with a deuterium flash lamp in our lifetime apparatus. Using an ultraviolet interference filter (Optics Technology, Palo Alto) to isolate a narrow band centered at 290 \text{ nm}, we obtained \( \tau = 21.8 \) nsec, which is not significantly different from \( \tau = 22.1 \) nsec obtained by exciting directly into the DNSA absorption band with the nitrogen flash lamp. Had the excited state of tryptophan persisted for, say, 2 nsec before transfer of energy, a larger value of \( \tau \) would have been detected.

It should be pointed out that the over-all energy transfer efficiency (85\%) from tryptophan to DNSA is higher than the degree of quenching of tryptophan fluorescence (73\%), which is also due to energy transfer. The simplest explanation for this difference is that the quantum efficiencies of the various tryptophan residues are not identical and that DNSA is situated in such a manner that energy transfer is more probable from those tryptophans which are relatively less fluorescent. In a simple model system in which all donor groups were identical, Latt, Cheung, and Blout (39) showed that the degree of fluorescence quenching by an energy acceptor group was equal to the degree of energy transfer. With regard to the possibility that the tryptophans in a protein may be heterogeneous, it is known that the fluorescence due to tryptophan varies in yield in different proteins and is markedly affected by the environment (40). An alternative explanation which cannot be wholly discounted at present is that the binding of DNSA causes a conformational change which influences the quantum yield of tryptophan emission.

**Nature of Binding Site**—The site occupied by DNSA appears to be quite specific for unsubstituted sulfonamides. DNSA can be displaced from carbonic anhydrase by other sulfonamides such as ethoxzolamide, benzenesulfonamide, and acetazolamide (1, 3, 4-thiazole-2-acetamido-5-sulfonamide). On the other hand, attempts to detect complexes of this enzyme with DNS-glycine, DNS-1-glutamic acid, DNS-1-proline, and DNS-1-tryptophan gave negative results in terms of protein fluorescence quenching, ligand fluorescence enhancement, or increase in polarization of visible fluorescence. These compounds are known to form highly fluorescent complexes with proteins such as serum albumin, apomyoglobin, and apheresomoglobin (8, 11, 14); however, they lack the basic primary sulfonamide structure needed to bind to carbonic anhydrase.

The changes in DNSA emission properties upon binding to the enzyme are shown in Fig. 5 summarized in Table I. At pH 7.4, free DNSA has a weak fluorescence with a peak at 580 \text{ nm} and a quantum yield of 0.055. In complex with carbonic anhydrase, DNSA emits maximally at 468 \text{ nm} with a yield of 0.84. The 112 \text{ nm} blue shift and the 15-fold quantum yield increase require comment.

In a recent study of the DNS-amino acids, it was shown that their emission spectra have peaks near 580 \text{ nm} in water and move toward shorter wave lengths in hydrophobic organic solvents (14). In dioxane, for instance, it was found that DNS-tryptophan had a peak at 500 \text{ nm}. Upon binding of DNS-amino acids such as DNS-L-proline to serum albumin, the peak of the spectrum of the visible fluorescence varied with the species of albumin tested and ranged from 486 to 502 \text{ nm}. The effects of solvents and protein solutions on the fluorescence of DNS-amino acids were interpreted in terms of a high sensitivity of the compounds to the dielectric constant of the environment (14). DNSA has the same chromophoric group as the DNS-amino acids, and, by spectral measurements in dioxane-water mixtures, we have found that DNSA shows similar solvent shifts in its emission. Furthermore, DNSA binds to bovine serum albumin, and the fluorescence spectrum is given in Fig. 6. The quantum yield of the fluorescence excited at 340 \text{ nm} is 0.64, and the peak is at 500 \text{ nm}. It is clear, then, that the hydrophobic nature of the binding site can account for part of the blue shift exhibited by CA(DNSA). However, the emission of CA(DNSA) is considerably bluer than that of the corresponding bovine serum

### Table I

<table>
<thead>
<tr>
<th>Compound</th>
<th>Exciting wave length</th>
<th>( \lambda_{\text{max}} ) of emission</th>
<th>Quantum yield</th>
<th>( \tau )</th>
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<tr>
<td>Carbonic anhydrase</td>
<td>290 336</td>
<td>0.11</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td>CA(DNSA)</td>
<td>290 336</td>
<td>0.04</td>
<td>9.4</td>
<td></td>
</tr>
<tr>
<td>CA(DNSA)</td>
<td>290 408</td>
<td>0.71</td>
<td>21.8</td>
<td></td>
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<tr>
<td>DNSA, pH 7.4</td>
<td>320 508</td>
<td>0.84</td>
<td>22.1</td>
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<tr>
<td>DNSA, pH 12</td>
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<td>0.86</td>
<td>4.0</td>
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<tr>
<td>DNS-CA</td>
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<td>0.090</td>
<td>22.0</td>
<td></td>
</tr>
<tr>
<td>BSA(DNSA)</td>
<td>345 500</td>
<td>0.64</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Fluorescence decay times were measured in the nanosecond flash fluorometer with suitable Corning or interference filters to give excitation at the wave lengths listed, which were used for the determination of quantum yields.
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Fig. 5. Corrected emission spectra of DNSA and CA(DNSA). The curves have been adjusted so that the areas underneath the lines are proportional to the quantum yields (which are listed in Table I). The spectra are of CA(DNSA) (○—○) excited at 310 μm, and of 1.0 × 10⁻⁴ M DNSA in 0.02 M potassium phosphate buffer, pH 7.4 (●—●), and in 0.01 M KOH (□—□). The solution used for the CA(DNSA) fluorescence was the same as that used for the absorption spectrum in Fig. 4.

FIG. 6. Emission spectra of DNS-containing proteins. The spectrum of CA(DNSA) is replotted from Fig. 5 for comparison. The curves are normalized to the same height, although the quantum yields (Table I) are different. The emission of BSA(DNSA) was obtained by 340 μm excitation of a solution described in Table I. The spectrum of DNS-CA, 3 × 10⁻⁵ M, was obtained by 335 μm excitation. Band width of emission monochromator, 12 μm.

albumin complex, in spite of the fact that albumin has a very hydrophobic binding site (10, 41). Therefore, yet another factor must be involved in determining the visible fluorescence spectrum of CA(DNSA).

We suggest that such a factor may be the loss of a proton from DNSA, i.e., the process ArSO₂NH₂ → ArSO₂NH⁻ + H⁺. Some of the evidence from other work that sulfonamides are bound to carbonic anhydrases in the form ArSO₂NH⁻ is considered under "Discussion." In addition, the following spectral characteristics of DNSA are consistent with loss of a proton upon binding to the enzyme. It is evident from Fig. 4 that the absorption spectrum of DNSA is markedly blue-shifted on binding to carbonic anhydrase. A difference spectrum was obtained with 6 × 10⁻³ M carbonic anhydrase in the reference cell and the same solution plus 3 × 10⁻³ M DNSA in the sample cell; the results showed bound DNSA to have an absorption maximum at 309 to 310 μm with ε = 4.50 × 10⁴ M⁻¹ cm⁻¹. In contrast, free DNSA has a peak at 323 μm (Fig. 4). A blue shift in the absorption spectrum of DNSA can also be induced by raising the pH, and we found that the spectrum of this compound in 0.01 M KOH had a peak at 310 μm with ε = 5.59 × 10⁴ M⁻¹ cm⁻¹. The blue shift in absorption at high pH, which was reversible, seems clearly to be due to ionization of the sulfonamide moiety. The process is accompanied by a blue shift in the fluorescence emission spectrum, with the peak changed from 580 μm to 540 μm (Fig. 5) and the quantum yield rising from 0.055 to 0.085. It might be expected, therefore, that ionization of the sulfonamide group on binding to carbonic anhydrase would involve a fluorescence blue shift of similar magnitude. Loss of a proton from DNSA on binding to the enzyme could thus account for the difference in position between the emission spectra of CA(DNSA) and BSA(DNSA) shown in Fig. 6.

The absorption blue shift shown by DNSA in alkali allowed us to study the ionization in more detail by titrating and monitoring the appearance of ionized DNSA by the increase in absorption at 305 μm (Fig. 7). The pKₐ of the -SO₂NH₂ group was found to be 9.79. Some compounds are stronger acids in their excited states; the subject of photoionization or ejection of protons from excited states has been reviewed recently by Wehry and Rogers (42). Since Lagunoff and O'ttolenghi (18) have shown that the protonated dimethylamino group of 5-dimethylaminonaphthalene-1-sulfonic acid at low pH was a much stronger acid in the excited singlet state, we examined the question whether the sulfonamide group of excited DNSA ionized more readily than the ground state. Fluorometric titration of DNSA performed by monitoring the appearance of ionized sulfonamide at 500 μm showed exactly the same pKₐ as spectrophotometric titration (Fig. 7). One can conclude, therefore, that if ejection of a proton from excited state DNSA occurs the process must be much
method used by Weber (46) to obtain rotational relaxation times utilizes proteins covalently labeled with fluorescent dyes. With the explicit assumptions that (a) the labeling is random and (b) the electronic oscillators of the ligand with respect to the axes of the protein ellipsoid (49). While such a criticism might be considered "random," as was assumed in the derivation of Equation 2 (46). The spectra of DNSA shift in the opposite direction, a finding which suggests that differences in ground and excited state acidities of the -SO₂NH₂ group are, in fact, not great.

The ionization of the sulfonamide group does not seem to be incompatible with the emission blue shift due to the transfer of DNSA from aqueous to hydrophobic environments. This was confirmed by investigation of the emission spectra of DNSA in solutions containing different proportions of dioxane and aqueous KOH. The effects of solvent and ionization of the sulfonamide group on the emission spectrum appear to be more or less additive. For instance, it was found that in 90% dioxane 10% 1 M KOH the emission maximum of DNSA was at 490 mp, whereas in 90% dioxane-10% 0.1 m potassium phosphate, pH 7.4, the peak was at 502 mp.

Inhibition of Carbonic Anhydrase by DNSA—In order to test the effect of DNSA on the activity of carbonic anhydrase, we employed the esterase assay with the substrate, p-nitrophenyl acetate (43). In the absence of inhibitor, linear Lineweaver-Burk plots were obtained, with Kₘ = 4 × 10⁻⁸ M and Vₘₐₓₚ = 2.5 × 10⁵ moles per min per mole of enzyme in 0.02 M potassium phosphate buffer, pH 7.4. DNSA proved to be a powerful inhibitor. The inhibitor constant, Kᵢ, as defined by Armstrong et al. (20), was obtained by their method, and found to be 4 × 10⁻⁷ M. The inhibition was apparently noncompetitive, because Vₘₐₓ was decreased at all inhibitor concentrations used.

It should be recalled that, although the inhibition of DNSA and other sulfonamides have been found by classical techniques to be noncompetitive (20, 44, 45), it has been pointed out that the inhibitors may still actually bind to the catalytic site (7). The apparently noncompetitive nature of the inhibition could be due to a relatively slow rate of release of inhibitor. One of our observations that supports this possibility is that the visible absorption spectrum of the CA(DNSA) complex appears only slowly when ethoxzolamide is added to the solution. The kinetics of the displacement of DNSA is currently under study, and preliminary results suggest that the obliteration of CA(DNSA) fluorescence by ethoxzolamide has a half-time of the order of 3 to 4 sec.

Rotational Relaxation Time of Carbonic Anhydrase—The method used by Weber (46) to obtain rotational relaxation times utilizes proteins covalently labeled with fluorescent dyes. With the explicit assumptions that (a) the labeling is random and (b) the macromolecule rotates as a rigid entity, the mean harmonic rotational relaxation time, ρ₀, can be obtained by measurements of the fluorescence polarization at different temperatures and viscosities. The Perrin-Weber equation (46) is

\[
\frac{1}{P} - \frac{1}{P₀} = \left( \frac{1}{P₀} - \frac{1}{3} \right) \left( 1 + \frac{3\tau}{\rho₀} \right)
\]

(2)

where P and P₀ are the fluorescence polarizations with linearly polarized exciting light in media of finite and infinite viscosities, respectively, and τ is the fluorescence decay time. The relaxation time, ρ₀, obtained this way is often compared with the relaxation time, ρ₀, of the anhydrous sphere of equivalent molecular weight obtained from (46)

\[
ρ₀ = \frac{3\eta V}{RT}
\]

(3)

where η is the viscosity, V the molecular volume, R the gas constant, and T the absolute temperature. Equation 2 is used in conjunction with measurements of P at different values of T/η to give ρ₀; plots of 1/P with respect to T/η are extrapolated to infinite viscosity to give P₀, and T is independently measured.

For the CA(DNSA) complex, a fluorescence decay time at 25°C of 22.1 nsec was obtained, and measurements of the polarization of fluorescence as a function of temperature yielded the Perrin-Weber plot shown in Fig. 8. At 25°C, the relaxation time is thus 28.9 nsec. From Equation 3, ρ₀ = 24.3 nsec, with the assumption that the molecular weight is 30,000 and the partial specific volume is 0.73, so that the ratio ρ₀/ρ₀ is 1.10. For most proteins rotating as rigid ellipsoids, the relaxation time ratio ρ₀/ρ₀ has been found to be between about 1.7 and 2.0 (47). The low ρ₀/ρ₀ ratio observed here for CA(DNSA) suggests that the molecule has a low degree of asymmetry, and there is some indirect evidence for this. The measurements of intrinsic viscosity by Armstrong et al. (20) on human carbonic anhydrase B suggested that the protein must be nearly spherical. Work of Nyman and Lindegkog (15, 48) shows that there are a number of similarities between bovine carbonic anhydrase B and the human enzymes; these similarities include aspects of the amino acid compositions, molecular weights, sedimentation coefficients, and affinities for zinc. Similarities in molecular shape might also be expected.

A possible criticism of the use of the fluorescence depolarization method in the case of a protein complex containing a single fluorescent group is that the labeling can in no sense be considered "random," as was assumed in the derivation of Equation 2 (46). When a single moiety is bound to a macromolecule, it is possible that the results will be influenced by preferential orientation of the electronic oscillators of the ligand with respect to the axes of the protein ellipsoid (49). While such a criticism might be applicable to relaxation time studies already reported with ANS bound to bovine serum albumin or to apomyoglobin and apo-
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FIG. 8. Perrin-Weber plot for the depolarization of CA(DNSA) fluorescence. A solution of $4.4 \times 10^{-4}$ M carbonic anhydrase containing $6 \times 10^{-4}$ M DNSA in 0.02 M potassium phosphate buffer was excited with polarized light at 320 nm, and the emitted light was analyzed with the monochromator set at 470 nm.

The data were analyzed by the C-E-I-R, Inc., on site computer service to determine the slope and intercept of the line defined by the points according to a mean least squares approximation.

hemoglobin (8, 9), the objection will be only minor in the case of bovine carbonic anhydrase, if, like the human enzyme (20), its shape is nearly spherical. Furthermore, the relaxation time was similar to those obtained with a conjugate prepared by reaction of carbonic anhydrase with DNS-chloride, as is discussed below.

Properties of DNS-CA Conjugate—It seemed desirable to compare the properties of CA(DNSA) with those of a conjugate of the enzyme which contained the same number of DNS-groups. The conditions found to yield such a conjugate are as follows. To 1 ml of 0.0 M NaHCO₃ containing 10 mg of carbonic anhydrase was added 0.1 ml of acetone containing 0.2 mg of DNS-chloride, and the mixture was stirred in an ice bath for 4 hours. The mixture was then dialyzed for 48 hours at 2° against 1 liter of 0.002 M potassium phosphate buffer, pH 7.0, containing 3 g of activated charcoal as a slurry (Darco). The dialysate was changed twice in this time. The dialyzed protein was free of unconjugated dye as determined by paper chromatography (50). The average degree of labeling was determined spectrophotometrically (17) and found to be 1.1 moles of DNS- per mole of protein; the absorption spectrum of the complex is shown in Fig. 9.

There were numerous differences between the conjugate and the complex, CA(DNSA). First, the absorption spectrum has a maximum at 335 nm for the DNS- band (Fig. 9); this is to be compared with a peak at 309 to 310 nm obtained from the difference spectrum of CA(DNSA) minus enzyme alone (cf. Fig. 4). Second, energy transfer from tryptophan to DNS- in the conjugate is only 10% efficient, as is shown by calculations based on the excitation and absorption spectra of Fig. 9. The data of Figs. 4 and 9 were used to calculate the transfer distance $R_0$ for the conjugate, and $R_0$ was found to be 22.8 A. The transfer distances for conjugate and complex are thus similar; the small difference was due to greater spectral overlap (higher $J_0$) in the case of the conjugate. Third, the quantum yield of tryptophan fluorescence in the conjugate was 0.09, compared with 0.11 in the unlabeled protein. In the conjugate, therefore, quenching by DNS- occurs to the extent of only 20% compared with 73% in CA(DNSA). Fourth, the fluorescence characteristics of the DNS- conjugate were quite dissimilar to those described for CA(DNSA). Instead of a blue fluorescence, the emission of conjugate appeared green under a ultraviolet light, and the corrected emission spectrum had a maximum at 533 nm (Fig. 6). The quantum yield was 0.12 (Table I), and the fluorescence decay time was 12.2 nsec in 0.02 M potassium phosphate buffer, pH 7.4. Fifth, although our data indicated that the enzyme containing bound DNSA is inactive, we found the DNS- conjugate to be 100% as active as the unlabeled enzyme in the esterase assay.

Calculation of Mean Effective Transfer Distance—The energy transfer data obtained with CA(DNSA) and DNS-CA permit calculation of a mean effective distance, $R$, between donor and acceptor groups. From transfer theory (34, 51),

$$1 - X = \frac{1}{(R_0/R)^n + 1}$$

where $X$ is the fraction of the absorbed photons which are transferred. In CA(DNSA), $X = 0.85$, $R_0 = 21.3$ A, and $R = 16.0$ A. For the conjugate, DNS-CA, $X = 0.10$, $R_0 = 22.8$ A, and $R = 32.9$ A. On the average, therefore, the tryptophans in the conjugate are about twice as far from the DNS- group as they are in the DNSA complex. An anhydrous sphere of 30,000 molecular weight and a specific volume of 0.73 would have a

FIG. 9. Energy transfer in a DNS conjugate of carbonic anhydrase. The absorption spectrum (---) of $2.4 \times 10^{-4}$ M DNS-CA and the excitation spectrum of the same solution diluted 10-fold (●---●) have been normalized at long wave lengths, where the two curves coincide. The excitation spectrum was obtained with an 8-nm excitation band width, the emission being observed at 540 nm.

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protein, the following results were obtained: Fig. 10A, in 0.02 M potassium phosphate buffer, pH 7.4 (A), the same buffer plus 10^{-4} M ethoxzolamide (B), and 0.1 M NaHCO_3 (C). The data were analyzed as in Fig. 8. Exciting wave length, 335 mp; emission was observed at 540 mp.

The present studies on the binding of fluorescent groups to bovine carbonic anhydrase B provide some direct spectral information bearing on the chemical nature of the sulfonamide-binding site. The very high quantum yield and the fluorescence spectrum of bound DNSA suggest that the site is shielded from solvent interactions and has a highly hydrophobic character. As pointed out above, the fact that activity studies with sulfonamides have given Lineweaver-Burk curves suggestive of noncompetitive inhibition does not rule out the possibility that the sulfonamides bind at the site required for the catalysis of CO_2 hydration. If the active site and the sulfonamide-binding site are indeed identical, we are left with the mild paradox that water reacts at or near a hydrophobic site.

The reason for the strong binding and inhibitory power of aromatic sulfonamides has been unclear for some time, since sulfonamides are only weak ligands for Zn^{2+}. This study suggests that the sulfonamide-binding site consists of two sections, one of which promotes the dissociation of a proton from the —SO_NH_2 group, and the other, a hydrophobic binding region which attracts the aromatic portion of the sulfonamide. Aside from the spectral evidence provided here for dissociation of the sulfonamide group, there is reasonably good kinetic evidence supporting such a scheme. Miller, Dessert, and Robin (3) postulated that ionization of the sulfonamide group was necessary for attachment to carbonic anhydrases because the inhibitory power of a series of sulfonamides was related to the pK_a compounds having more acidic —SO_NH_2 groups being in general more potent inhibitors. More recently, the inhibition of bovine carbonic anhydrase by benzenesulfonamide was followed by stop-flow techniques as a function of pH. An inflection in the pH-activity curve in the region of the sulfonamide pK_a again indicated that dissociation of a proton from the inhibitor occurs on binding (4).

A hydrophobic character of the sulfonamide-binding site has not previously been found by chemical techniques. It is of interest, however, that the hydrophobic portion of the binding site is clearly not able by itself to cause significant binding of hydrophobic ligands such as ANS or the DNS-amino acids.

If one can assume that the aromatic sulfonamide inhibitors bind to carbonic anhydrase through two groups (via a hydrophobic bond involving the aromatic nucleus and also through a bond involving the ionized sulfonamide moiety), a ready explanation for the large association constants for these inhibitors is at hand. The inhibitors may be likened to bidentate ligands capable of forming chelates with transition metal ions. Schwarzzenbach (52) pointed out that the formation constant for a chelate containing a bifunctional ligand is almost always much larger than the corresponding constant for the formation of a complex containing two monofunctional ligands. This "chelate effect" has been analyzed in terms of entropic and statistical factors favoring the stability of the chelate (52).

The energy transfer from tryptophan to DNSA is much more...
efficient than has been observed hitherto in any DNS-containing protein system. For instance, Stryer (36) studied the energy transfer from tryptophan to DNS in conjugates of chymotrypsinogen, a protein having a molecular weight of 24,000. When 5.5 DNS-groups were attached to the protein by reaction with DNS-chloride, 74% of the photons absorbed by tryptophan residues were transferred to the dye groups. These results are to be contrasted with the data here reported for the larger CA (DNSA) complex, in which energy transfer was 85%, with only 1 DNS-group. The critical transfer distance, R0, for the tryptophan-DNS-pair calculated by Stryer (36) is about the same as that found here for either CA(DNSA) or the DNS-conjugate of the enzyme. Therefore, the much higher degree of energy transfer in CA(DNSA) must be due to nearness to or favorable orientation in the tryptophan residues, or both.

In this regard, we should point to some other evidence concerning the location of the tryptophans and the sulfonamide-binding site. Although much of this evidence was derived with human carbonic anhydrase C, that enzyme has many similarities (molecular weight, amino acid composition, zinc-binding affinity, activity, and so forth) with bovine carbonic anhydrase B. Optical spectroscopy experiments by Riddiford (53) on the human enzyme suggest that the tryptophans are buried in the interior of the molecule. Further, Urry and Eyring (54) suggest that in human carbonic anhydrase at least 2 tryptophans may be in fixed juxtaposition, from evidence obtained by optical rotatory dispersion in the ultraviolet region.

Very recently, Fridborg et al. (6) published the results of a study at 5.5 Å resolution on the x-ray scattering of crystals of human carbonic anhydrase C containing the inhibitor acetonylmercuric sulfanilamide. Even at this relatively low resolution, it was possible to make several observations. Zinc was bound in the center of the molecule near the carboxyl terminus of the polypeptide chain. The inhibitor occupied part of a deep crevice containing the zinc and was bound to the metal ion through the sulfonamide group. The benzene ring of the inhibitor was seen to lie in a narrow slot. If this picture has validity also for bovine carbonic anhydrase B, the x-ray data would confirm the fluorescence evidence that the sulfonamide-binding site is well shielded and is in the interior of the protein.

The validity of Förster's theory of energy transfer has been tested and essentially confirmed by studies on models containing donor and acceptor groups in the same molecule (e.g., References 39, 55-57). Latt, Cheung, and Blout (39) studied energy transfer from the 1-naphthoyl group to anthracene-9-carbonyl in a rigid molecule and obtained rather direct evidence that the mutual orientation of the donor-acceptor pair affected transfer efficiency. Similar tests of Förster's theory have been impractical in proteins, but if further x-ray crystallographic data are forthcoming on carbonic anhydrases it may be possible to know the positions of the tryptophans with great accuracy and to correlate this information with energy transfer data such as those reported in this work.

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REFERENCES


Combination of Bovine Carbonic Anhydrase with a Fluorescent Sulfonamide
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