

Lack of Effect of the Length of Oligoglycine- and Oligo(ethylene glycol)-Derived para-Substituents on the Affinity of Benzenesulfonamides for Carbonic Anhydrase II in Solution

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Abstract: Using ^1H NMR spectroscopy, values of T_2 have been determined for the methylene protons of the oligoglycine moieties of para-substituted benzenesulfonamides having structures $\text{H}_2\text{NO}_2\text{SC}_6\text{H}_4\text{CO}(\text{Gly})_n\text{OH}$ ($n = 1-6$) bound at the active site of bovine carbonic anhydrase II (CA, EC 4.2.1.1). These values have been correlated with measurements of dissociation constants of these complexes, in order to infer motion of these ligands when bound to the enzyme. Motion of glycines 1-3 (those closest to the aryl ring) is hindered by their proximity to the protein; motion of glycines 4-6 is relatively unhindered. Despite the restriction to motion inferred for glycines 1-3, the values of K_d for the six compounds ($n = 1-6$, 1-6) are indistinguishable within experimental uncertainty ($\pm 20\%$): K_d in μM (n) 0.30 (1); 0.26 (2); 0.33 (3); 0.37 (4); 0.37 (5); 0.34 (6). There is, therefore, an unexpected compensation of the loss in conformational entropy on binding by another contributor to the free energy.

Introduction

We (and many others) are interested in developing procedures that will make it possible to design tight-binding inhibitors of enzymes.¹⁻⁴ The most widely used approach to the development of tight-binding inhibitors is to modify the structure of a known substrate or ligand.⁵ We are exploring a variant of this approach (outlined in Figure 1),⁶ in which we retain a constant structure for the inhibitor (the "primary" inhibitor, P, in Figure 1) and try to find a secondary ligand (S) that binds close to (but not at) the active site. Connecting P and S by a linker L would generate a new inhibitor PLS, whose affinity for the protein might be larger than that of P. Equation 1 relates the free energy of binding of

$$\Delta G_{\text{PLS}} = \Delta G_{\text{P}} + \Delta G_{\text{S}} + \Delta G_{\text{L}} \quad (1)$$

$$\Delta G_{\text{L}} = \Delta H_{\text{L}} - T\Delta S_{\text{L}} \quad (2)$$

PLS formally to that of P and S. If L is correctly designed, PLS will bind more tightly than P alone (that is, $\Delta G_{\text{PLS}} < \Delta G_{\text{P}}$) although probably less tightly than the maximum that would be obtained by simply adding the free energies of binding of P and S.⁷ The difference between $\Delta G_{\text{P}} + \Delta G_{\text{S}}$ and ΔG_{PLS} (represented by ΔG_{L} ; eqs 1 and 2) is a sum of contributions to both enthalpy and entropy. Contributions to ΔG_{L} can include interactions between L and the protein that can be either unfavorable or favorable, and unfavorable modification of the dissociation constants of P and S due to

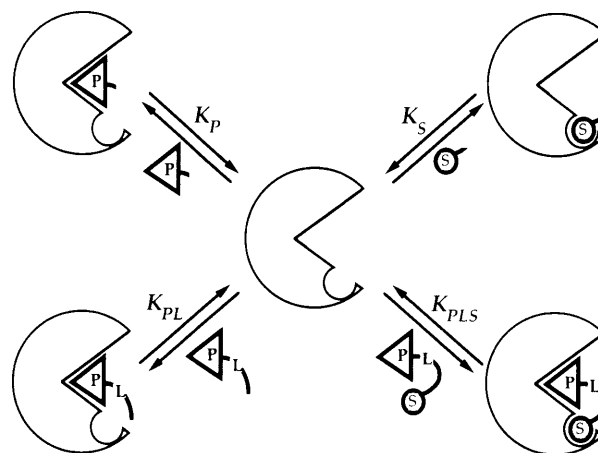


Figure 1. Bivalent tight-binding ligand for a receptor benefiting from two enthalpically favorable interactions. The affinity of the PLS inhibitor possessing both the primary (P) and secondary (S) recognition element linked by an appropriate linker (L) will be greater than that of either element alone, so long as L does not interact strongly in an unfavorable manner on binding.

their attachment to L.⁸ Even if these terms are all zero, there may be an unfavorable entropic term due to decreasing the degrees of conformational freedom of L on simultaneous binding of P and S.^{9,10}

Our efforts to design tight-binding inhibitors based on the PLS motif represent an attempt to increase the area of molecular surface of the inhibitor in contact with the protein, while retaining the desirable structural features of P and without paying an unacceptable price in free energy for the linking group (especially in unfavorable conformation entropy). Identifying the structure S, designing L, and defining the best method for connecting P

(8) Schweitzer-Stenner, R.; Licht, A.; Luscher, I.; Pecht, I. *Biochemistry* **1987**, *26*, 3602-3612.

(9) Portoghese, P. S.; Ronsisvalle, G.; Larson, D. L.; Takemori, A. E. *J. Med. Chem.* **1986**, *29*, 1650-1653. Cram, D. J. *Angew. Chem., Int. Ed. Engl.* **1986**, *25*, 1039-1057.

(10) Williams, D. H.; Cox, J. P. L.; Doig, A. J.; Gardner, M.; Gerhard, U.; Kaye, P. T.; Lal, A. R.; Nicholls, I. A.; Salter, C. J.; Mitchell, R. C. *J. Am. Chem. Soc.* **1991**, *113*, 7020-7030.

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(1) Bartlett, P. A.; Shea, G. T.; Telfer, S. J.; Waterman, S. *Spec. Publ.—R. Soc. Chem.* **1989**, *78*, 182-196. Bedell, C. R. *Chem. Soc. Rev.* **1984**, *13*, 279-319. Knowles, J. R.; Widlanski, T. S. *J. Am. Chem. Soc.* **1989**, *111*, 2299-2300.

(2) Dean, P. M. *Molecular Foundations of Drug-Receptor Interactions*; Cambridge University Press: Cambridge, U. K., 1987.

(3) *Crystallographic and Modeling Methods in Molecular Design*; Bugg, C. E., Ealick, S. E., Eds.; Springer-Verlag: New York, 1990.

(4) Kresge, A. J. *Acc. Chem. Res.* **1987**, *20*, 364-370.

(5) Dugas, H. *Bioorganic Chemistry*; Springer-Verlag: New York, 1989; pp 96-102. Kaplan, A. P.; Bartlett, P. A. *Biochemistry* **1991**, *30*, 8165-8170.

(6) Broom, A. D. *J. Med. Chem.* **1989**, *32*, 2-7. Portoghese, P. S.; Larson, D. L.; Sayre, L. M.; Ronsisvalle, G.; Tam, S. W.; Takemori, A. E. *J. Med. Chem.* **1986**, *29*, 1855-1861.

(7) Portoghese, P. S.; Lipkowski, A. W.; Takemori, A. E. *J. Med. Chem.* **1987**, *30*, 238-239.

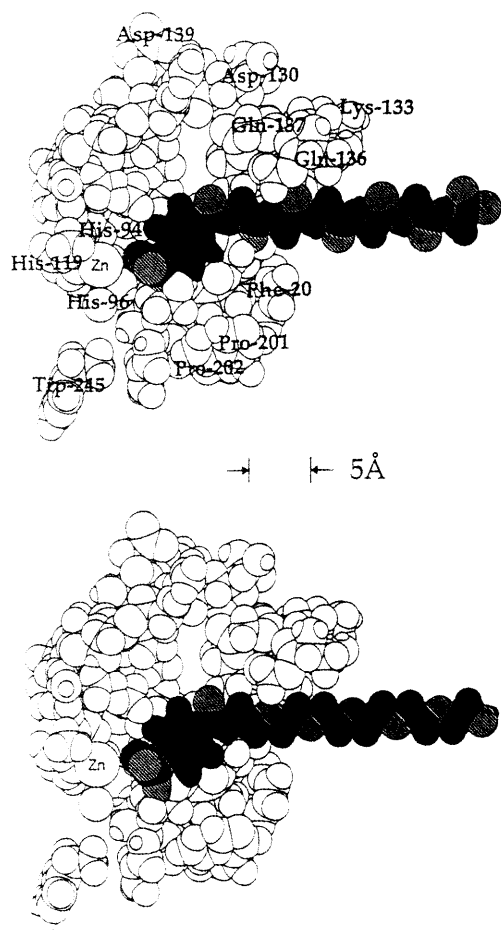


Figure 2. Plane section through the active site of carbonic anhydrase, with $\text{H}_2\text{NO}_2\text{SC}_6\text{H}_4\text{CONH}(\text{Gly})_6\text{OH}$ (upper) and $\text{H}_2\text{NO}_2\text{SC}_6\text{H}_4\text{CONH}(\text{EG})_6\text{OH}$ (lower) superimposed for scale (structural data are taken from ref 13).

and S to L represent significant problems in molecular design. This paper describes an exploratory study directed primarily toward examining the interaction between protein and two plausible classes of linking groups L: oligoglycines and oligo(ethylene glycols). We have used bovine carbonic anhydrase II (CA, EC 4.2.1.1) as a model protein and 4-sulfamoylbenzoic acid ($\text{H}_2\text{NO}_2\text{SC}_6\text{H}_4\text{CO}_2\text{H}$) as P.

The interaction of CA with arenesulfonamides has been studied extensively.¹¹ Two plane sections through the CA binding site are shown in Figure 2, with van der Waals surfaces for $\text{H}_2\text{NO}_2\text{SC}_6\text{H}_4\text{CO}(\text{Gly})_6\text{OH}$ (6) and $\text{H}_2\text{NO}_2\text{SC}_6\text{H}_4\text{CONH}(\text{EG})_6\text{OH}$ (7-OH) superimposed for scale.¹² This binding site has been described as a roughly conical cleft on the surface of the enzyme.¹³ The divalent zinc ion at the base of the cleft is approximately 15 Å from the "surface" of the protein. The width of the binding site is such that there would seem to be little geometrically enforced van der Waals contact between the linkers L and the protein, and relatively little restriction to conformational freedom beyond $m = n = 3$.

(11) Baldwin, J. J.; Ponticello, G. S.; Anderson, P. S.; Christy, M. E.; Murcko, M. A.; Randall, W. C.; Schwam, H.; Sugrue, M. F.; Springer, J. P.; Gautheron, P.; Grove, J.; Mallorga, P.; Viader, M.-P.; McKeever, B. M.; Navia, M. A. *J. Med. Chem.* **1989**, *32*, 2510–2513. For reviews of inhibition of CA by sulfonamides, see: Coleman, J. E. *Annu. Rev. Pharmacol.* **1975**, *15*, 221–242. Maren, T. H. *Ann. N.Y. Acad. Sci.* **1984**, *429*, 569–579.

(12) Eriksson, A. E.; Jones, T. A.; Liljas, A. *Proteins: Struct., Funct., Genet.* **1988**, *4*, 274–282.

(13) For reviews of the structure and catalytic mechanism of CA, see: Pocker, Y.; Sarkanen, S. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1978**, *47*, 149–274. Lindskog, S.; Henderson, L. E.; Kannan, K. K.; Liljas, A.; Nyman, P. O.; Strandberg, B. In *The Enzymes*; Boyer, P. D., Ed.; Academic: New York, 1971; Vol. 5, pp 587–665. Silverman, D. N.; Lindskog, S. *Acc. Chem. Res.* **1988**, *21*, 30–36. Christianson, D. W. *Adv. Protein Chem.* **1991**, *42*, 281–355.

We have measured dissociation constants between CA and $\text{H}_2\text{NO}_2\text{SC}_6\text{H}_4\text{CO}(\text{Gly})_n\text{O}^- \text{K}^+$ and $\text{H}_2\text{NO}_2\text{SC}_6\text{H}_4\text{CONH}(\text{EG})_m\text{OR}'$ (1–6), $n = 1–6$; 7-OCH₃–12-OCH₃, $m = 1–5$, R' = CH₃; 7-OCH₂Ph–12-OCH₂Ph, $m = 1–6$, R' = CH₂Ph and have inferred the conformational freedom of the constituent groups of these potential linking groups L from values of T_2 obtained by ¹H NMR spectroscopy. Our interest was to correlate these two sets of data and to inquire if interactions between CA and L that are sufficient to inhibit motion of L are also sufficient to influence binding. The surprising conclusion from this work is that motion of L can apparently be restricted without influencing the ΔG of binding. That is, a restriction to the motion of the substituent large enough to influence T_2 need not influence the ΔG of binding.

Results

Synthesis of Ligands. We prepared oligoglycine- and oligo(ethylene glycol)-substituted arenesulfonamides by allowing the amine terminus of the appropriate oligomer to react with the *N*-hydroxysuccinimidyl ester of $\text{H}_2\text{NO}_2\text{SC}_6\text{H}_4\text{CO}_2\text{H}$ ($\text{H}_2\text{NO}_2\text{SC}_6\text{H}_4\text{CONHS}$).¹⁴ The oligoglycines were commercially available, and the oligo(ethylene glycol)-substituted amines either were commercially available ($m = 1$, R = CH₃) or were prepared by standard methods from the monoethers of the corresponding glycols ($m = 1–5$, R = CH₃ and $m = 1$, R = CH₂Ph are commercially available; $m = 2–6$, R = CH₂Ph were prepared by monoalkylation of the appropriate glycols). The methyl esters 1-OCH₃ and 3-OCH₃ were prepared either by coupling of the methyl ester of the peptide to $\text{H}_2\text{NO}_2\text{SC}_6\text{H}_4\text{CONHS}$ or by esterification of the free acid in methyl alcohol containing acidic ion-exchange resin.

Dissociation Constants. We measured dissociation constants using a competitive fluorescence-based assay.¹⁵ This assay is based on the fact that the quantum yield for fluorescence of 5-(dimethylamino)-1-naphthalenesulfonamide (DNSA), upon excitation at 320 nm, increases in going from aqueous solution to the hydrophobic cleft of CA. We (and others¹⁶) have modified the procedure developed by Chen and Kernohan by using light with a wavelength of 290 nm (an absorption minimum for DNSA) to excite the ten tryptophan residues of the protein.¹⁵ Energy is transferred from excited tryptophans ($\lambda_{\text{emiss}}^{\text{max}} = 320$ nm) to bound DNSA ($\lambda_{\text{abs}}^{\text{max}} = 326$ nm). The only signal detected at 460 nm on excitation at 290 nm is from CA·DNSA. This variation on the procedure of Chen and Kernohan reduces background signal from free DNSA in solution. Knowing the dissociation constant of CA·DNSA (we have measured $K_d = 0.38$ μM for bovine CA II at 25 °C; this value agrees with that reported in the literature¹⁵) and the total concentration of CA in a given solution, it is possible to use mass balance to calculate the concentration of CA bound to DNSA (CA·DNSA), bound to a nonfluorescent inhibitor (CA·Inh), and free in solution. The dissociation constant of CA·Inh (the inverse of the 1:1 binding constant, K_{11}^{inh}) was determined using a Scatchard plot of data from the titration, according to eq 3.¹⁷ Since this assay for binding involves a

$$\frac{[\text{CA}\cdot\text{Inh}]}{[\text{CA}]_{\text{tot}}\cdot[\text{Inh}]} = K_{11}^{\text{inh}} - K_{11}^{\text{inh}} \left[\frac{[\text{CA}\cdot\text{Inh}] + [\text{CA}\cdot\text{DNSA}]}{([\text{CA}]_{\text{tot}})} \right] \quad (3)$$

competition between a "probe inhibitor," DNSA, and the inhibitor of interest, Inh, values of saturation of the active site of CA by the inhibitor of interest that are less than the extent of initial saturation of CA with DNSA cannot be obtained (typically, 0.6).

The results from our fluorescence measurements are summarized in Table 1, and typical data are shown in Figure 3.

(14) Anderson, G. W.; Zimmerman, J. E.; Callahan, F. M. *J. Am. Chem. Soc.* **1964**, *86*, 1839–1842.

(15) Chen, R. F.; Kernohan, J. C. *J. Biol. Chem.* **242**, 1967, 5813–5823.

(16) Ponticello, G. S.; Freedman, M. B.; Habecker, C. N.; Lyle, P. A.; Schwam, H.; Varga, S. L.; Christy, M. E.; Randall, W. C.; Baldwin, J. J. *J. Med. Chem.* **1987**, *30*, 591–597.

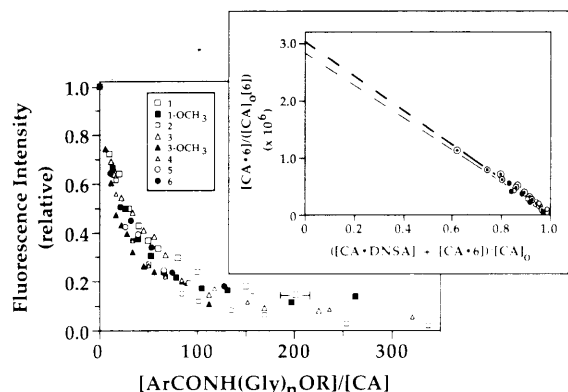


Figure 3. Titrations of CA·DNSA complex with $\text{H}_2\text{NO}_2\text{SC}_6\text{H}_4\text{CONH}(\text{Gly})_n\text{OR}$ (1–6, R = H or CH_3) at 298 K. The extent of inhibition was monitored by observing the decrease in fluorescence on addition of the nonfluorescent inhibitor. The uncertainty of each y-value is approximately the size of the symbol for each datum. At low values of x , the uncertainty in x is approximately the size of each point; for large x -values, a typical error bar is shown. Inset: Scatchard analyses of titrations of CA·DNSA with $\text{H}_2\text{NO}_2\text{SC}_6\text{H}_4\text{CONH}(\text{Gly})_6\text{OH}$ (6). Four data sets are shown. The theoretical minimum value of x for this type of Scatchard plot, obtained from a competitive binding experiment, is 0.6 (see text for details) ($[\text{CA}] = 50 \text{ nM}$; $20 \text{ mM KH}_2\text{PO}_4$ (pH 7.5)).

Oligoglycine-derived benzenesulfonamides all bind to CA with dissociation constants between 0.26 and $0.37 \mu\text{M}$. Derivatives of oligo(ethylene glycol) have dissociation constants that are 2- to 4-fold (for those that terminate in methoxyl) and 10-fold smaller (for those that terminate in benzyl) than those of the oligoglycine derivatives.¹⁸

^1H NMR Spin Echo Spectroscopy of CA· $\text{H}_2\text{NO}_2\text{SC}_6\text{H}_4\text{CO}(\text{Gly})_n\text{OR}$. ^1H NMR spin echo spectra from the titration of CA with 3–6 are shown in Figures 4, and values of T_2 are summarized in Table 2.¹⁹ From the data shown in Figure 4a, we inferred that the mobility of the first three glycine units in CA·6 was restricted relative to that of the last three; the last three had values of T_2 similar to those of 6 free in solution.

(17) Derivation of Scatchard plot of data from fluorescence assay.

$$K_{11}^{\text{inh}} = \frac{[\text{CA}\cdot\text{Inh}]}{[\text{CA}][\text{Inh}]} \quad (4)$$

But

$$[\text{CA}] = [\text{CA}]_{\text{tot}} - [\text{CA}\cdot\text{Inh}] - [\text{CA}\cdot\text{DNSA}] \quad (5)$$

So,

$$K_{11}^{\text{inh}} = \frac{[\text{CA}\cdot\text{Inh}]}{([\text{CA}]_{\text{tot}} - [\text{CA}\cdot\text{Inh}] - [\text{CA}\cdot\text{DNSA}])[\text{Inh}]} \quad (6)$$

Rearranging,

$$K_{11}^{\text{inh}} \left[1 - \frac{[\text{CA}\cdot\text{Inh}] + [\text{CA}\cdot\text{DNSA}]}{([\text{CA}]_{\text{tot}})} \right] = \frac{[\text{CA}\cdot\text{Inh}]}{[\text{CA}]_{\text{tot}}[\text{Inh}]} \quad (7)$$

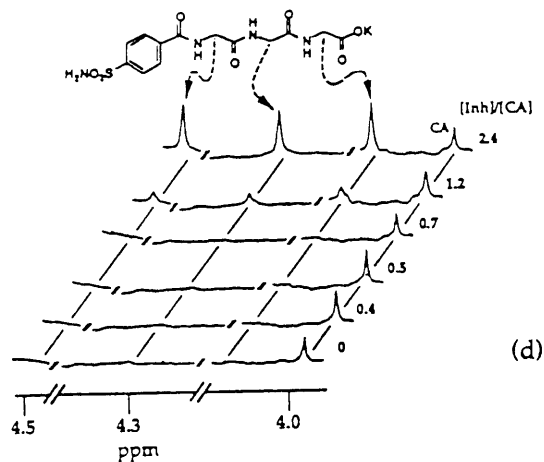
or

$$K_{11}^{\text{inh}} - K_{11}^{\text{inh}} \left[\frac{[\text{CA}\cdot\text{Inh}] + [\text{CA}\cdot\text{DNSA}]}{([\text{CA}]_{\text{tot}})} \right] = \frac{[\text{CA}\cdot\text{Inh}]}{[\text{CA}]_{\text{tot}}[\text{Inh}]} \quad (8)$$

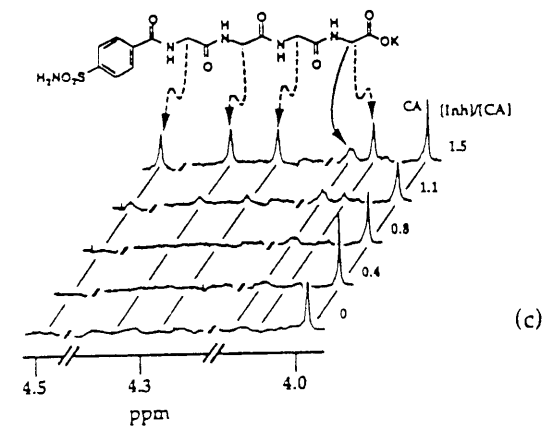
$[\text{CA}]_{\text{tot}}$ is known directly, as are $[\text{DNSA}]_{\text{tot}}$ and $[\text{Inh}]_{\text{tot}}$ at each point in the titration of CA·DNSA with Inh. $[\text{CA}\cdot\text{DNSA}]$ is obtained from the fluorescence signal, since only this species affords signal under the experimental conditions that we use. Because we know the total concentration of DNSA used in any given titration, from the dissociation constant of CA·DNSA, we can calculate $[\text{CA}]$, and from eq 2, $[\text{CA}\cdot\text{Inh}]$. Finally, the concentration of free inhibitor, $[\text{Inh}]$, is obtained from mass balance, since we know $[\text{Inh}]_{\text{tot}}$ at each point in the titration.

(18) We have determined the dissociation constants of several methyl and benzyl esters of oligopeptide-substituted benzenesulfonamides. These values are consistent with the observation in the EG series, that addition of a benzyl group to an inhibitor results in enhanced recognition of that inhibitor by CA, if the benzyl group is within or close to the binding cleft of the protein when the inhibitor is bound (R = CH_2Ph K_d in μM (n): 0.071 (2); 0.075 (3); 0.21 (4). R = CH_3 K_d in μM (n): 0.26 (1); 0.32 (3)).

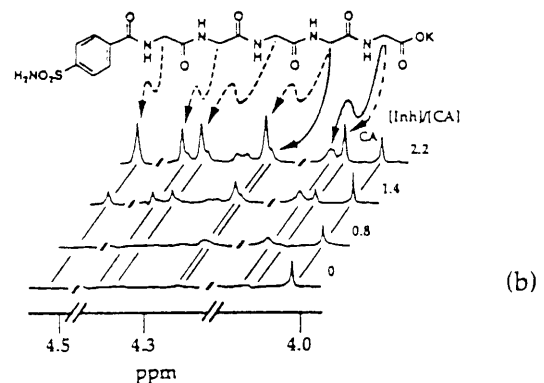
(19) The concentration of protein used in these experiments was 0.4 mM. At this concentration, given the dissociation constants in Table 1, >95% of all inhibitor added to the solution of CA (up to 1 equiv) is bound.



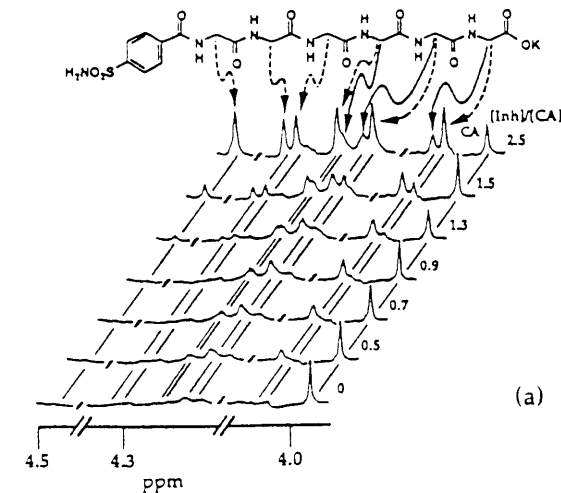
(d)



(c)



(b)



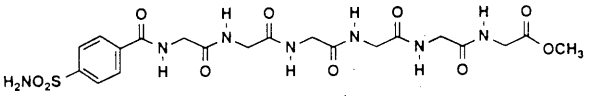
(a)

Figure 4. ^1H NMR spin echo spectra of CA at 0.4 mM titrated with $\text{H}_2\text{NO}_2\text{SC}_6\text{H}_4\text{CONH}(\text{Gly})_n\text{OH}$: (a) $n = 6$; (b) $n = 5$; (c) $n = 4$; (d) $n = 3$. Solid lines indicate resonances due to protein-bound inhibitors; dashed lines indicate resonances due to free inhibitors. The resonance labeled "CA" is present in all solutions containing CA (Inh = inhibitor; $20 \text{ mM KD}_2\text{PO}_4$ ("pH" 7.5)).

Table 1. Dissociation Constants of Oligoglycine- and Oligo(ethylene glycol)-Linked Arylsulfonamides (ArCONH(Gly)_nOK, ArCONH(EG)_mOCH₃, and ArCONH(EG)_mOCH₂Ph) from Bovine Carbonic Anhydrase II

oligomer length (n, m)	K _d , μM ^a		
	(Gly) _n O-K ⁺	(EG) _m OCH ₃	(EG) _m OCH ₂ Ph
1	0.30 ^b	0.10	0.028
2	0.26	0.13	0.033
3	0.33 ^b	0.16	0.041
4	0.37	0.21	0.036
5	0.37	0.21	0.036
6	0.34		0.054

^a Uncertainties in these values are ±20%. ^b 1-OCH₃ inhibits human carbonic anhydrase III with K_d = 0.063; μ3-OCH₂Ph inhibits human carbonic anhydrase II with K_d = 0.075 μM; the crystal structures of these enzyme-inhibitor complexes are reported in ref 24.

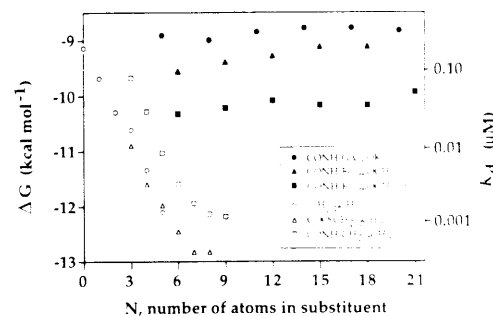
Table 2. Transverse Relaxation Times (T₂, ms), Determined by ¹H NMR Spin Echo Spectroscopy at 323 K, of Protons of Eight Inhibitors Complexed with CA^a


Cmpd	T ₂ (ms)					
1	s					
1-OCH ₃	s					200
2	s	s				
3	s	s	s			
3-OCH ₃	s	s	s			330
4	s	s	s	120		
5	s	s	s	120	120	
6	s	s	s	120	120	120
6 (soln)	230	230	230	230	230	230

^a Columns refer to methylene or methyl groups, referenced to the (Gly)₆ derivative shown. Values of T₂ less than 25 ms could not be precisely determined due to background signal from protein at the values of the delay τ corresponding to those relaxation times (protons belonging to CA have T₂ ~ 25 ms): These times are indicated in the Table by s (for small). The uncertainties in these values are ±40 ms.

When the molar ratio of **6** to CA was 0.9, we were able to observe three distinct resonances in the spectrum that we could assign to NHCH₂CO groups. These peaks had unequal line widths, with the most upfield resonance (which corresponds to the sixth glycine unit) being the narrowest. This observation is not inconsistent with the values of T₂ listed in Table 2, since the experimental uncertainties in these values are large. The trend that we have observed in line widths, however, does agree with our expectation that more distal parts of the inhibitors should be less restricted when bound to CA than proximal residues. Examination of the points in the titration up to 1 equiv of inhibitor added showed a monotonic increase in the size of the three peaks; after 1 equiv of inhibitor was added, these three resonances did not increase further in intensity, and six new resonances with equal line widths appeared. These narrow peaks grew steadily in intensity as more inhibitor was added. The shorter relaxation times of the resonances of CA-bound inhibitors are consistent with the restriction of motion of CH₂ groups by the binding cleft of the protein.²⁰ We conclude that for CA-**6** the motion of three glycine groups is restricted by the protein and that three are

(20) Sommerville, L. E.; Henry, G. D.; Sykes, B. D.; Hartshorne, D. J. *Biochemistry* **1990**, *29*, 10855–10864. Sharp, R. R.; Frasch, W. D. *Biochemistry* **1985**, *24*, 5449–5454. Navon, G.; Lanir, A. *J. Magn. Reson.* **1972**, *8*, 144–151. Lanir, A.; Navon, G. *Biochemistry* **1971**, *10*, 1024–1032.

**Figure 5.** Free energies of binding (ΔG) of a series of oligomer-substituted benzenesulfonamide derivatives (data represented by open symbols is from ref 22). N is the number of atoms past the benzenesulfonamide ring that each inhibitor extends (for the purposes of this graph, we have counted the benzyl group as one atom): N = 8 for H₂NO₂SC₆H₄CONH(Gly)₂OK, for example.

moving relatively freely (compare values of T₂ of spins due to bound CH₂ units and those of free inhibitors, listed in Table 2). By direct analogy to the assignments of resonances of free inhibitor, and in accord with their chemical shifts, we assigned the resonances as indicated in Figure 4a.

From the results of the titration of CA with **6**, we expected that **5** would have two CH₂ peaks that were sharp enough to be observable and that **4** would show only one peak when bound. These expectations were both realized (Figure 4b and c). We were not able to observe any methylene resonances in the spectra of complexes of CA with H₂NO₂SC₆H₄CO(Gly)_nOR for n ≤ 3 (Figure 4d; n = 3).²¹ Due to the multiplicity of the resonances of molecules in the ethylene glycol series, and because not all these resonances were resolved, we were unable to obtain any quantitative data for their complexes with CA. We did acquire spectra of CA-**11-OCH₃**, CA-**7-OCH₂Ph**, and CA-**8-OCH₂Ph**; these data are included in Figure 6 in the supplementary material.

Discussion

King and Burgen have reported a relevant study of the inhibition of CA by several classes of alkyl-substituted 4-sulfamoylbenzoic acid derivatives (H₂NO₂SC₆H₄X(CH₂)_nCH₃; X = CH₂, CO₂, CONH).²² These studies correlated the structure and association constants (and rates of binding) to CA of derivatives of benzenesulfonamide bearing alkyl chains of varying length. They concluded that inhibitors bearing longer alkyl chains bind more tightly to CA (Figure 5) and that the differences in dissociation constants reflect primarily differences in values of k_{off}; that is, inhibitors having longer hydrophobic substituents dissociate more slowly from the active site than inhibitors with shorter and less hydrophobic substituents.

(21) We obtained additional information regarding interactions between oligomer-substituted arylsulfonamides and the binding cleft of CA from comparison of the spin echo spectra of its complexes with H₂NO₂SC₆H₄CONH(Gly)₁OCH₃ (**1-OCH₃**) and H₂NO₂SC₆H₄CONH(Gly)₃OCH₃ (**3-OCH₃**). In both of these spectra, we observed resonances of methyl groups of enzyme-bound inhibitors. The detection of a resonance due to a methyl group in the spectrum of an enzyme-bound inhibitor, even in the case where the protons giving rise to this resonance should be within the binding cleft (up to 14 Å from the bottom of the cleft in the case of CA-**1-OCH₃**), is consistent with the work of Lanir and Navon,²⁰ who demonstrated that the relaxation properties of methyl groups are unique. Even when a methyl group is well within the active site, none of the spins due to protein residues in the cleft are likely to effect rapid relaxation of the magnetization of the methyl protons, the primary mechanism for relaxation of methyl protons being intramolecular. The T₂ of the methyl protons in CA-**1-OCH₃** is less than that of free **1-OCH₃** (200 ms vs 380 ms, Table 2), corresponding to a decrease in the overall rotational correlation time of this moiety due to its being within the active site; some component of the motion of this group is relatively restricted in the bound state. The methyl group in CA-**3-OCH₃**, on the other hand, may extend 20 Å from the bottom of the cleft, and these protons have a T₂ closer to that of the methyl group of free **3-OCH₃** (330 ms vs 380 ms, Table 2), suggesting that virtually no interactions occurred between the methyl group and CA.

(22) King, R. W.; Burgen, A. S. V. *Proc. R. Soc. London, B* **1976**, *193*, 107–125.

The results described by King and Burgen differ qualitatively from those we have obtained in the oligoglycine and oligo(ethylene glycol) series. They observed a substantial, monotonic increase in binding affinity with increasing length of *n*-alkyl chains; we observe little change with increasing length of (Gly)_{*n*} or (EG)_{*m*} (EG = ethylene glycol) chains. We attribute the increased affinity of the derivatives bearing longer alkyl chains to their interaction with the hydrophobic face of the binding cavity of CA. The relatively tight binding of the benzyl-terminated derivatives H₂NO₂SC₆H₄CO(EG)_{*m*}OCH₂Ph (Table 1) may be due to similar interactions, which are discussed in greater detail elsewhere.^{23,24}

The most unexpected aspect of this work is the insensitivity of the dissociation constants of the (Gly)_{*n*}- and (EG)_{*m*}-substituted arenesulfonamides to length. The NMR spectroscopic studies indicate that at least the portions of the oligoglycine chains close to the aryl group interact sufficiently strongly with the surface to restrict their motion; this inference is supported by crystallographic studies of CA·H₂NO₂SC₆H₄CO(Gly)₁OCH₃ (**1-OCH₃**) and CA·H₂NO₂SC₆H₄CO(Gly)₃OCH₂Ph (**3-OCH₂Ph**),²⁴ which show that the peptide chains of these molecules fold along the hydrophobic wall and interact with Lue-184 and -198 and Pro-201 and -202, for example. These interactions must contribute favorably to the *enthalpy* of binding but probably also contribute unfavorably to the *entropy* of binding, by restricting the conformational mobility of the chains. The most plausible rationalization of the observation that ΔG of binding is approximately invariant with chain length is that changes in ΔH and $T\Delta S$ coincidentally cancel. A qualitative discussion of this cancellation is outlined by Cappalunga Bunn et al.²⁴

These studies indicate, once again,²⁵ how difficult it is to predict influences of structure on binding. In the context of this study, both (EG)_{*m*} and (Gly)_{*n*} linkers appear to be relatively innocuous. A fortuitous cancellation of terms allows them to fit into the binding site of CA without excessive cost in free energy. They should, therefore, be suitable linkers with which to explore ligands PLS (Figure 1) for this enzyme, and initial studies of this type of ligand are reported elsewhere.²³ Our detailed understanding of the factors entering into these interactions is, however, sufficiently limited that extension of these results to a different enzyme would require independent experimental verification.

Experimental Section

General Methods. Carbonic anhydrase was obtained from Sigma (as a 1:4 mixture of type II bovine isozymes A and B) and was used without further purification. Starting materials for the syntheses of inhibitors were of the best grade available, from Aldrich, Fluka, Chemical Dynamics, Bachem Biochemicals, Kodak, or Parish Chemicals. Syntheses were carried out under nitrogen unless otherwise specified. Yields of inhibitors were not optimized. Silica gel was used for thin-layer and column chromatography. ¹H NMR experiments were carried out on a Bruker AM-500 spectrometer operating at 500 MHz for proton, with a dedicated ¹H probe. ¹³C spectra were obtained in Me₂SO-*d*₆, with a Bruker AM-400 spectrometer operating at 100 MHz for carbon. ¹H NMR data for compounds 1–6 are in Table 3; ¹³C NMR data, melting points, and elemental analyses (or, in some cases, high-resolution positive- or negative-ion FABMS) for 1–6 are in Table 4, and ¹H and ¹³C NMR data and elemental analyses (or, in some cases, high-resolution positive-ion FABMS) for 7–12 are in Table 5. These tables are in the supplemental material. Elemental analyses were performed by Spang.

***N*-Hydroxysuccinimidyl 4-Sulfamoylbenzenecarboxylate (H₂NO₂SC₆H₄CONHS).** Dicyclohexylcarbodiimide (10.0 g, 50 mmol) was added to 4-sulfamoylbenzoic acid (10.0 g, 50 mmol) and *N*-hydroxysuccinimide (6.4 g, 56 mmol) in 320 mL of *N,N*-dimethylformamide (DMF) at 0 °C. The white suspension of *N,N'*-dicyclohexylurea (DCU) was allowed to stir overnight and was filtered (9.4 g), after chilling to 0 °C. The filtrate

was concentrated to an oil. This crude product was washed with hot 2-propanol, affording an off-white solid (12.0 g, 80%, mp 174–176 °C): ¹H NMR δ 8.29 (d, 7.4, 2H), δ 8.07 (d, 7.4, 2H), δ 7.70 (s, 2H); ¹³C NMR δ 170.4, 161.3, 150.1, 131.2, 127.5, 127.0, 25.9. Anal. Calcd for C₁₁H₁₀N₂O₆S: C, 44.30; H, 3.38; N, 9.39. Found: C, 44.37; H, 3.47; N, 9.36.

General Procedure for the Synthesis of H₂NO₂SC₆H₄CO(Gly)_{*n*}OR (1–6). Inhibitors were prepared by standard procedures for the synthesis of short oligopeptides, by coupling of 1 equiv of the appropriate nucleophile to H₂NO₂SC₆H₄CONHS in 1:2 acetone/0.1 M KH₂PO₄ (pH 8), at 0.1 M concentration of nucleophile. The compound H₂NO₂SC₆H₄CO(Gly)₃OCH₃ (**3-OCH₃**) was prepared by stirring H₂NO₂SC₆H₄CO(Gly)₃OH (**3**) with acidic ion-exchange resin in methyl alcohol. In all cases, products were isolated by removal of the solvent *in vacuo* and purification of the residue either by flash column chromatography (in cases where the compounds were soluble in acetone or ethyl acetate) or by ion-exchange chromatography (for water-soluble products) or by exhaustive trituration of the crude material with water, acetone, and methanol. A typical synthetic procedure is provided below.

1-(((Glycylglycyl)glycyl)carbonyl)-4-sulfamoylbenzene (H₂NO₂SC₆H₄CO(Gly)₃ONa, **3).** To triglycine (0.19 g, 1.0 mmol) in 6.6 mL of 0.1 M KH₂PO₄ (pH 8) was added H₂NO₂SC₆H₄CONHS (0.30 g, 1.0 mmol) in 3.3 mL of acetone. The reaction mixture was stirred at room temperature for 2 h, at which point the product was isolated by removal of the solvent *in vacuo*. The residue was washed with 25 mL of cold H₂O and with 50 mL of hot acetone leaving **3** as a white solid (0.28 g, 75%).

General Procedure for the Synthesis of Methyl-Terminated Oligo(ethylene glycol)-Substituted Amides of 4-Sulfamoylbenzoic Acid (H₂NO₂SC₆H₄CONH(EG)_{*m*}OCH₃, *m* = 2–5, 8-OCH₃–11-OCH₃). The commercially available monomethyl ethers of *m* = 1–6 were converted to their tosylates (toluenesulfonyl chloride, pyridine, 0 °C, overnight), and the tosylates were displaced with sodium azide (DMF, 50 °C, 12 h). The crude azides were reduced with triphenylphosphine in tetrahydrofuran (THF) (room temperature, 12 h), followed by hydrolysis (room temperature, 12 h); the resulting amines were allowed to react with H₂NO₂SC₆H₄CONHS as described above to afford the desired amides, which were characterized by ¹H and ¹³C NMR and high-resolution FABMS or elemental analysis (Table 5). A typical synthetic procedure is provided below.

(2-Methoxyethyl)amide of 4-Sulfamoylbenzoic Acid (H₂NO₂SC₆H₄CONH(EG)₁OCH₃, 7-OCH₃). To H₂NO₂SC₆H₄CONHS (0.3 g, 1.0 mmol) in acetone (4 mL) was added 2-methoxyethylamine (100 μ L, 1.1 mmol) in 20 mM KH₂PO₄ (pH 8; 8 mL). The reaction mixture was stirred at room temperature for 2 h, at which point the product was isolated by removal of the solvent *in vacuo* and recrystallization of the crude white powder from 10 mL of hot, distilled, deionized H₂O, affording **7-OCH₃** (clear plates, 64 mg (25%); second crop 53 mg (20%).

General Procedure for the Synthesis of Benzyl-Terminated Oligo(ethylene glycol)-Substituted Amides of 4-Sulfamoylbenzoic Acid (H₂NO₂SC₆H₄CONH(EG)_{*m*}OCH₂Ph, *m* = 1–6, 7-OCH₂Ph–12-OCH₂Ph). The monoalkoxides of the commercially available glycols *m* = 2–6 were prepared by deprotonation (1 equiv of sodium hydroxide, THF). Each of these suspensions was alkylated with 1 equiv of benzyl bromide (room temperature, 12 h). The resulting crude benzyl ethers (together with the commercially available *m* = 1 derivative) were purified by flash column chromatography. The tosylates of the benzyl ethers were prepared as described above and were displaced with sodium azide in DMF (50 °C, 12 h). The crude azides were reduced with triphenylphosphine in THF (room temperature, 12 h), followed by hydrolysis (room temperature, 12 h), and the resulting amines reacted with H₂NO₂SC₆H₄CONHS as described above to afford the desired amides, which were characterized by ¹H and ¹³C NMR and high-resolution FABMS or elemental analysis (Table 5). A typical synthetic procedure is provided below.

(2-(Benzyloxy)ethyl)amide of 4-Sulfamoylbenzoic Acid (H₂NO₂SC₆H₄CONH(EG)₁OCH₂Ph, 7-OCH₂Ph). 2-(Benzyloxy)ethanol (5.0 g, 33 mmol) was converted to 2-(benzyloxy)ethylamine (1.68 g, 34% overall yield) via the sequence of reactions described above. This amine (0.18 g, 1.16 mmol), in 0.1 M KH₂PO₄ (pH 8; 7 mL), was added to H₂NO₂SC₆H₄CONHS (0.30 g, 1.0 mmol) in acetone (3 mL). The solution was stirred at room temperature for 2 h, at which point **7-OCH₂Ph** was isolated by filtration (clear plates, 0.22 g (57%); second crop 0.13 g (34%).

Dissociation Constants. Fluorescence spectrophotometry was carried out with a Perkin-Elmer Model MPF-4 spectrofluorimeter, equipped with a cooling bath to regulate the temperature in the cuvette. All dissociation constants were determined at 25 °C.

(23) Jain, A.; Whitesides, G. M.; Alexander, R. S.; Christianson, D. W. *J. Am. Chem. Soc.*, submitted.

(24) Cappalunga, Bunn, A. M.; Alexander, R. S.; Christianson, D. W. *J. Am. Chem. Soc.*, following paper in this issue.

(25) Morgan, B. P.; Scholtz, J. M.; Ballinger, M. D.; Zipkin, I. D.; Bartlett, P. A. *J. Am. Chem. Soc.* **1991**, *113*, 297–307.

Protein was added from 20–30 μM stock solutions to 3.00 mL of 20 mM KH_2PO_4 (pH 7.5) to a final concentration of 50–100 nM. Stock solutions of inhibitors were prepared gravimetrically at ca. 20 mM in $\text{Me}_2\text{SO}-d_6$ and were calibrated by the following procedure: the stock solutions were diluted 10-fold into solutions of 1.84 mM *N,N*-dimethylformamide (DMF) in $\text{Me}_2\text{SO}-d_6$. We obtained ^1H NMR spectra of each of these samples, with an adequate delay (2 s) between pulses to allow complete relaxation of all spins. By comparing the integrals of the peaks due to inhibitor and to DMF, we accurately determined the concentrations of the stock solutions of inhibitors. These stock solutions were serially diluted to obtain titrant solutions for fluorescence experiments of 1.0 or 0.1 mM. We added inhibitor to the fluorescence cells with a Hamilton syringe whenever the volume was less than 20 μL , or else with an Eppendorf pipette. The fluorescence from each of these solutions was measured at 460 nm, upon excitation at 290 nm. The data (corrected for dilution by titrant volume) were analyzed via Scatchard plots.¹⁷ Titrations were routinely carried out to $\geq 99\%$ saturation of the receptor by the ligand.

^1H NMR Spin Echo Spectroscopy. ^1H NMR spin echo experiments on CA-sulfonamides were executed at 323 K. The pulse sequence used was a simple Hahn's echo ($\pi/2-\tau-\pi-\tau$).²⁶ Before each experiment, we determined the 90° and 180° pulse widths and the longitudinal (T_1) and transverse (T_2) relaxation times of the spins of the protein, at 323 K. The typical value for the 90° pulse width, measured after tuning the dedicated ^1H probe to D_2O solvent, was 7 μs . To apply the spin echo experiment to this study, it was essential to know the minimum delay time (τ) for suppression of the spins of protein, otherwise signals from relatively mobile moieties in the complexes may have been suppressed. We found 40 ms to be the best choice for this delay time. For quantitation of T_2 , data were obtained at ≥ 13 delay times ranging from 20 to 900 ms. The values of T_2 reported in Table 2 represent calculations from the specific delay time at which that particular resonance disappeared following acquisition

with the spin echo pulse sequence. The error value of ± 40 ms is an estimate based on evaluation of multiple sets of data collected for each inhibitor. We also found that precise temperature regulation during the experiment was essential, since the chemical shifts of resonances of bound inhibitor are temperature sensitive (up to 0.02 ppm/deg) and peaks are therefore artificially broadened by fluctuations in temperature over the course of each acquisition (25 min). We collected data from samples equilibrated for 20 min in the probe, with temperature equal to 323 ± 0.1 K (this uncertainty is the resolution of the instrument's thermocouple). Solutions of protein were prepared in 20 mM KD_2PO_4 ("pH" 7.5) by dissolution of a known amount of protein in a known volume of buffer (typically 65 mg in 5.0 mL of 20 mM KH_2PO_4 (pH 7.5)), lyophilization several times, with D_2O washes, and finally, dissolution in D_2O (5.0 mL, 99.98% D). The concentration of protein used in each experiment was determined spectrophotometrically from a 0.020 to 3.020-mL dilution of the solution to be used in that experiment ($\epsilon_{280} = 5.7 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$).¹⁵ Solutions of inhibitors were calibrated by NMR, as described above. Inhibitors were added to CA with Hamilton syringes.

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Supplementary Material Available: Tables of ^1H and ^{13}C NMR data and elemental analyses for compounds 1–12 and a figure showing the ^1H NMR spin echo spectra of CA (7 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

(26) Sanders, J. K. M.; Hunter, B. K. *Modern NMR Spectroscopy*; Oxford: Oxford, U.K., 1989; pp 249–252.