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BENZENESULFONAMIDE-PEPTIDE CONJUGATES AS PROBES FOR SECONDARY BINDING SITES NEAR THE ACTIVE SITE OF CARBONIC ANHYDRASE

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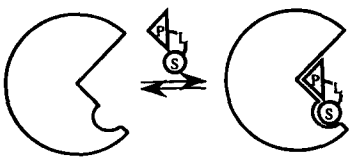
Abstract: Libraries of N-(4-sulfamoylbenzoyl)oligoglycines terminated with different L-amino acids were screened to identify tight binding inhibitors of human carbonic anhydrase II. Inhibitors terminated with hydrophobic amino acids showed significant enhancements in binding compared to the corresponding glycine derivatives. No enhancements were observed due to polar interactions.

Introduction

We and others are developing a procedure for identifying tight-binding ligands for proteins based on exploiting secondary non-covalent interactions near, but not in, the natural binding site. This procedure involves



fixing the structure of a primary binding element (P) and varying the structure of secondary binding elements (S) as well as the linking groups (L) that position these secondary elements relative to the primary element.[1] Assuming the linker does not interact with the protein, the binding energy of the bivalent ligand will equal the sum of the two individual binding energies minus the entropic free energy lost due to the restriction of the conformational space available to the linker ($\Delta G_{PLS} = \Delta G_P + \Delta G_S - T\Delta S_L$). This approach has several advantages: (i) the rapid synthesis of a large number of trial compounds; (ii) the compatibility with the use of libraries of functional groups; (iii) the ability to probe areas on the surface of the protein distant from the active site; (iv) the identification of secondary binding sites that can be utilized in the rational design of more conventional inhibitors. This paper is a continuation of our exploration of this strategy; it continues our work (and the work of others) using the benzenesulfonamide-derived inhibitors of human carbonic anhydrase II (HCAII, EC 4.2.1.1) as a model system.[1-4]

The Zn(II) ion of HCAII is at the bottom of a conical cleft that is roughly 15 Å deep.[5] The cleft can be divided into two sides; one hydrophilic, one hydrophobic. Previous studies have located two hydrophobic secondary binding sites on the hydrophobic face (Figure 1).[1] One site, in close proximity to the sulfonamide binding site, is a groove defined primarily by Phe-131, Val-135, Leu-198, Pro-202, and Leu-204; this site is responsible for the fact that hydrophobic amides of 4-sulfamoylbenzoic acid (SB), **1**, bind 20-400 times more tightly than 4-sulfamoylbenzamide. The association of the benzyl group of 4-sulfamoylbenzoic acid benzylamide (SB-benzylamide), **2**, with this site has been confirmed by X-ray crystallography.[3] The second site is responsible for the fact that the benzyl ester of the triglycine linked benzenesulfonamide (SB-$(Gly)_3$-OBz), **3**, binds roughly 5 times better than the free acids (**4**).[1] Although the benzyl group of this class of compounds is too

mobile in the binding pocket to be observed by X-ray crystallography,[3] molecular modeling suggests that the benzyl group of the tri(glycine) benzyl ester derivative binds near the lip of the active site in a hydrophobic site defined by Pro-201 and Phe-20.[4] Interestingly, the oligoglycine linkers appear to make no contribution to the binding constant, despite evidence from NMR spectroscopy and X-ray crystallography that they associate with the surface of the active site; a favorable enthalpy of binding seems to be exactly compensated by an unfavorable entropy of binding, due to loss of conformational freedom.[2]

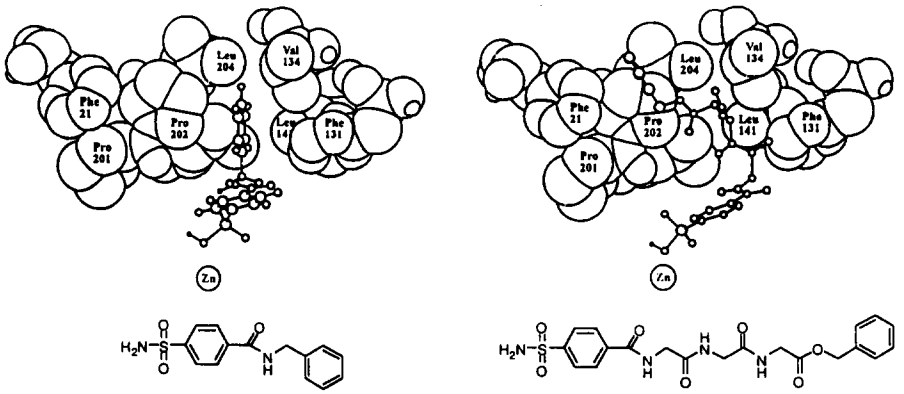


Figure 1. The location of SB-benzylamide and SB-$(Gly)_3$-OBz on the hydrophobic face of the HCAII binding pocket.

This paper describes the screening of libraries of N-(4-sulfamoylbenzoyl)oligoglycines terminated with different L-amino acids (SB-$(Gly)_n$-AA-OH, n= 0-3, 5). The objectives are: (i) to identify new secondary binding sites; in particular, to identify polar secondary binding elements capable of associating with polar groups on the protein. (ii) to define better the geometric requirements of the two previously identified hydrophobic binding patches. (iii) to help to understand how these hydrophobic interactions stabilize the protein-ligand complexes.

Results

Measurement of Dissociation Constants. We prepared inhibitors of the form SB-$(Gly)_n$-AA-OH by the reaction of an excess of the L-amino acids with the N-hydroxysuccinimide (NHS) ester of the corresponding SB-$(Gly)_n$-OH in a mixture of water and DMF buffered to pH 8.5 with sodium bicarbonate;[6,7] under these conditions HPLC showed that less than 10% of the NHS esters were lost to hydrolysis. The reaction products were tested, without further purification, for binding affinity to HCAII at pH 7.5 by the competitive displacement of dansylamide, an HCAII inhibitor that shows enhanced fluorescence when bound to HCAII.[2,8] Table 1 show the results. Selected inhibitors were purified to homogeneity and characterized by NMR spectroscopy and HRMS;[9] these purified inhibitors gave values of K_d which differed from the values of K_d measured for the crude materials by less than 20%.

The series of inhibitors with no linker (n = 0) showed a range of dissociation constant (K_d) covering three orders of magnitude, with, as expected, a clear preference for amino acids with hydrophobic side chains. Comparison of the different hydrophobic side-chains shows aliphatic groups contribute more to the strength of

Table 1. Dissociation Constants of the 4-Sulfamoylbenzoic Acid Derivatives (SB-$(Gly)_n$-AA-OH) to HCAII at 37 °C.

				K_d (nM)			
AA[a]	MSA (Å^2)[b]	ΔG_p[c]	RT (min)[d]	n = 0	n = 1	n = 2	n = 3
Asp	65	-8.72	14.0	1100	420	400	300
Glu	88	-6.81	14.5	530	500	372	371
Asn	67	-6.64	18.6	380	290	270	290
Gln	89	-5.54	18.9	140	240	270	280
Ser	43	-3.40	19.6	240	280	290	360
Thr	66	-2.57	20.1	53	320	330	330
Gly	10	0.94	20.5	310	290	230	390
Ala	36	1.81	21.0	120	170	250	300
Aph	130		21.4	64	330	210	190
Arg	116	-14.92	22.2	220	190	300	290
Pro	71		22.2	230	250	340	330
Val	82	4.04	22.6	15	140	260	290
Bgl	101		23.4	9	150	240	210
Met	84	2.35	23.7	10	160	160	220
Ile	101	4.92	24.3	9	160	230	230
Leu	103	4.92	24.7	9	130	210	210
Nle	105		25.3	5	140	140	190
Phe	124	2.98	25.7	13	140	140	200
Mty	153		26.4	27	170	170	170
Nph	148		26.6	22	150	130	160
Fph	130		27.4	17	130	120	140
Cph	142		28.0	15	100	94	96
Nal	171		29.1	17	130	160	160

[a] Natural L-amino acids (AA) are listed according to their standard three letter abbreviations. The following unnatural L-amino acids were also tested: p-aminophenylalanine (Aph), t-butylglycine (Bgl), norleucine (Nle), O-methyltyrosine (Mty), p-nitrophenylalanine (Nph), p-fluorophenylalanine (Fph), p-chlorophenylalanine (Cph), and 3-(2-napthyl)-alanine (Nal). Lys, His, Tyr, and Cys gave more than one major product from the coupling reaction and were not tested. [b] The MSA of the side chains were calculated for energy minimized structures of the amino acids as described in ref 5. [c] Free energy (Kcal/mol) of transfer of the amino acid side chain from dilute solution in cyclohexane to water at pH 7 (Radzicka, A.; Wolfenden, R. *Biochemistry* **1988**, *27*, 1664). [d] HPLC retention times (RT) of 4-dimethylaminoazobenzene-4'-sulfonyl chloride labeled amino acids on a C18-silica column in a gradient of acetonitrile in pH 6.5 acetate buffer (the synthesis of the labeled amino acids is described in Chang, J. Y.; Knecht, R.; Braun, D.G. *Methods in Enzymol.* **1983**, *91*, 41).

binding than aromatic groups, despite the larger molecular surface areas (MSA)[10] of the set of aromatic side-chains that were tested. The tightest binding inhibitor of the n = 0 series, the norleucine conjugate (K_a = 5 nM, MSA of side-chain = 105 Å^2) bound almost three times better than the p-chlorophenylalanine conjugate (K_a = 14 nM, MSA of side-chain = 142 Å^2). This preference for aliphatic groups has also been observed in the partition coefficients for the transfer of amino acid side-chain groups from water to cyclohexane;[11] the larger hydrophobic surface areas of the aromatic groups are counterbalanced by a higher polarizability that stabilizes these groups in polar solvents relative to aliphatic groups.[12] The order of binding affinities may also reflect steric constraints in the groove which forms this secondary binding pocket; increasing the size of the side-chain of the inhibitors with aromatic amino acids actually decreased the binding affinity.

For inhibitors with $(Gly)_n$ linkers of length n = 1-3, there is again a preference for side-chains with hydrophobic character (Table 1), although it is smaller by a factor of 10^2 than for the n = 0 series. We presume that this preference is due to interactions with the previously identified hydrophobic surface defined by Phe-20 and Pro-201.[4] There are two interesting features of this secondary binding site: first, the binding energies are relatively independent of the length of the linker chain, indicating a lack of strong geometric constraints on the

binding interaction. Second, in contrast to the n = 0 series of inhibitors, the tightest binding inhibitors of these series are terminated by amino acid with large aromatic side-chains. The order of binding affinities is similar to the order of elution of N-labeled amino acids from a C18-silica reverse phase HPLC column. The results are consistent with the known mobility of the benzyl group of SB-$(Gly)_3$-OBz in the active site,[4] and suggest a loose association of the secondary binding elements to a large, roughly two-dimensional hydrophobic surface.

Measurement of Kinetics of Dissociation. Taylor et al. noted a strong correlation between the rates of association of 4-sulfamoylbenzene derivatives with HCAII and their octanol-water partition coefficients, as well as with their affinity for apocarbonic anhydrase (the protein lacking an active site Zn(II)).[13] Based on these results, they proposed a two-step mechanism for binding: a partitioning of the inhibitor into the active site region (primarily due to hydrophobic interactions), followed by coordination of the weakly associated inhibitor to the active site Zn(II) to give the final complex.

$$\text{CA + Inh} \underset{}{\overset{K_1}{\rightleftharpoons}} \text{[CA•Inh]} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{[CA-Inh]} \qquad (1)$$

Assuming the partitioning is fast compared to the formation and cleavage of the sulfonamide-Zn bond, Taylor et al. pointed out that $k_{on} \approx K_1 k_2$ and $k_{off} \approx k_{-2}$. The measured value of k_{off}, therefore, reflects primarily the rate of cleavage of the coordination bond to the active site Zn. Stabilization of the orientation of the coordinated sulfonamide by a secondary binding interaction should result in a decrease in k_{off}.

We prepared and purified the glycine, norleucine, and p-chlorophenylalanine derivatives of the n = 0 and n = 3 series and measured the values of K_d and k_{off} for these inhibitors.[9] We determined the values of k_{off} by measuring the decrease in tryptophan fluorescence after displacement of the inhibitors with a large excess of diaminoazobenzenesulfonamide (an inhibitor that quenches the fluorescence of tryptophans near the active site of HCAII). Table 2 shows the results.

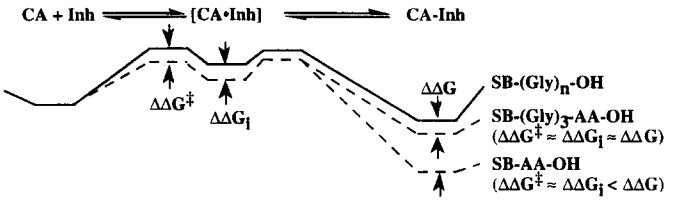
Table 2. Kinetic Constants for the Binding of 4-Sulfamoylbenzoic Acid Derivatives to HCAII.[a]

Inhibitor	K_d (nM)	k_{off} (s^{-1})	k_{on} (10^4 $M^{-1}s^{-1}$)
SB-Gly-OH	330	0.120	36
SB-Nle-OH	5	0.011	220
SB-Cph-OH	14	0.022	160
SB-$(Gly)_3$-Gly-OH	360	0.090	25
SB-$(Gly)_3$-Nle-OH	220	0.093	42
SB-$(Gly)_3$-Cph-OH	99	0.084	85

[a] Inhibitors were prepared as described for Table 1 and were further purified by recrystallization or flash chromatography. Values of K_d were measured as described for Table 1. Values of k_{on} were calculated from the measured values of K_d and k_{off} ($K_{on} = K_d k_{off}$).

When compared to SB-Gly, SB-Nle has both a 10-fold slower value of k_{off} and a 5-fold faster value of k_{on} (calculated by $k_{on} = k_{off}/K_d$); the large contribution from k_{off} suggests that the secondary binding interaction does indeed stabilize the orientation of the SB group in the active site, and makes it more difficult to break the sulfonamide-Zn(II) bond (i.e., the final bound state is stabilized relative to a transition state resembling the intermediate bound state). Alternatively, one could describe the increase in k_{off} as indicating that the energy of the hydrophobic interaction is greater in the final bound state than in the intermediate state. In either case, the evidence strongly suggests the hydrophobic side-chains are bound tightly in the final bound state. In contrast, the

terminal amino acids of the n = 3 series are less likely to influence the stability of the coordination to the Zn, because of the length of the linker between the primary and secondary binding elements, and because of the high mobility of the secondary binding element in the binding pocket (the hydrophobic interaction has equal influence on the energy of the weakly associated intermediate bound state and the tightly bound final bound state). The data for this series confirm this prediction; the observation that the terminal amino acid did not affect the k_{off} suggests that the primary and secondary binding elements act relatively independently.



Contributions of Polar Side-Chains to Binding. Table 1 shows that inhibitors with charged or primarily polar side-chains had binding constants similar to, or worse than, the corresponding glycine terminated inhibitor. The only examples of significant (> factor of two) improvements were SB-Thr (K_d = 53 nM) and SB-Gln (K_d = 140 nM), compared to SB-Gly (K_d = 310 nM). These improvements, however, may in fact be due to non-polar interactions; these inhibitors bind significantly better than other inhibitors with the same polar functional groups but with smaller hydrophobic surface areas -- SB-Ser (K_d = 240 nM) and SB-Asn (K_d = 380 nM). The entropic cost of orienting a group on a flexible linker into the correct geometry for forming hydrogen bonds with the protein may be too high to allow the effective use of polar interactions using this strategy without detailed analysis and design.[14]

SUMMARY AND CONCLUSION

Inhibitors prepared by linking amino acids to the C-terminal end of SB-oligo(glycines) showed enhanced binding only if the side-chain of the amino acids were capable of participating in hydrophobic interactions with the walls of the binding pocket. The geometric constraints required for stabilization of the bound complexes through hydrogen bonds were apparently too restrictive to see enhancements of binding resulting from the attachment of polar secondary binding elements to the SB group through flexible linker units. Hydrophobic amino acids directly attached to the SB group led to large enhancements in binding affinity. The magnitude of the enhancement, the effect of side-chain on off-rates, and the steric preference against large aromatic side-chains all suggest a tight binding of the hydrophobic side-chains to a geometrically well defined hydrophobic binding site. Hydrophobic amino acids linked to the SB group through an oligo(glycine) linker also enhance binding to HCAII. These interactions, however, lead to only small increases in binding affinity and suggest a weak association of the hydrophobic group with a large unstructured hydrophobic surface. The inefficiency of this interaction might be explained by one or more of the following reasons: (i) only a small percentage of the available surface area of the secondary binding element is actually in contact with the protein surface. (ii) entropic costs due to freezing the available conformational space of the linker limit the energy available from binding. (iii) the free energy of the interaction of hydrophobic groups with the secondary binding site, on the basis of the

surface area of the hydrophobic groups in contact with the binding sites, is small (i.e., the surface has limited hydrophobic character).

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6. Synthesis of the SB-$(Gly)_n$-NHS, n = 0-3: The SB-$(Gly)_n$-OH free acids (1 mmol, ref 2) were combined with NHS (1 mmol) and DCC (1 mmol) in 10 mL of DMF. The solutions were stirred overnight and were then concentrated to give oils. Recrystalization (n = 0-2) or trituratation (n = 3) in isopropanol gave white solids. Data for SB-$(Gly)_3$-NHS: ^{1}H NMR: (400 MHz, DMSO-d_6) δ 8.98 (t, 1H), 8.52 (t, 1H), 8.34 (t, 1H), 8.03 (d, 2H), 7.90 (d, 2H), 7.49 (s, 2H), 4.28 (d, 2H), 3.95 (d, 2H), 3.79 (d, 2H), 2.81 (s,4H); ^{13}C NMR: (100 MHz, DMSO-d_6) δ 170.01, 169.71, 169.21, 166.31, 165.60, 146.40, 136.85, 128.09, 125.61, 42.72, 41.73, 38.23, 25.46.
7. Crude synthesis of SB-$(Gly)_n$-AA-OH, n = 0-3: A solution containing the SB-$(Gly)_n$-NHS (2 μmol) in 50 μL of DMF was added to an aq solution containing the AA (5 μmol) and sodium bicarbonate (25 μmol. The resulting solution was incubated for 24 h at room temperature.
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9. Purification of selected SB-$(Gly)_n$-AA-OH, n = 0,3: The crude reaction mixtures were acidified to a pH of 1.5 with 1N HCl and then concentrated to dryness. The crude compounds of the n = 3 were recrystalized from water to give white solids. The crude compounds of the n = 0 series were first taken up in water and extracted several times into ethyl acetate. The combined organic phases were then dried over magnesium sulfate and concentrated. Recrystalization from water (Gly) or ethyl acetate-pentane (Nle, Cph) gave white solids. Data for SB-p-Cl-Phe-OH: ^{1}H NMR: (400 MHz, DMSO-d_6) δ 8.92 (d, 1H), 7.92 (d, 2H), 7.87 (d, 2H), 7.48 (s, 2H), 7.32 (s, 4H), 4.62 (m, 1H), 3.19 (dd, 1H), 3.04 (dd, 1H); ^{13}C NMR: (100 MHz, DMSO-d_6) δ 172.70, 165.28, 146.44, 137.05, 136.64, 131.09, 130.97, 128.13, 127.96, 125.63, 54.00, 35.54. HRMS calcd for $C_{16}H_{15}N_2O_5SCl$ $(M+Na)^+$ 314.0935; found $(M+Na)^+$ 383.0468. Data for SB $(Gly)_3$-Nle-OH: ^{1}H NMR: (400 MHz, DMSO-d_6) δ 8.97 (t, 1H), 8.28 (t, 1H), 8.09 (t, 1H), 8.03 (m, 3H), 7.89 (d, 2H), 7.48 (s, 2H), 4.14 (m, 1H), 3.93 (d, 2H), 3.74 (d, 4H), 1.56-1.69 (m, 2H), 1.25 (m, 4H), 0.83 (t, 3H); ^{13}C NMR: (100 MHz, DMSO-d_6) δ 173.50, 169.18, 168.96, 168.59, 165.53, 146.37, 136.82, 128.02, 125.55, 51.76, 42.72, 42.07, 41.57, 30.73, 27.40, 21.68, 13.74. HRMS calcd for $C_{19}H_{27}N_5O_8S$ $(M+H)^+$ 486.1659; found $(M+H)^+$ 486.1644.
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14. Alternatively, the difficulty in finding polar secondary binding elements could be ascribed to the tendency of the peptide chains to associate with the hydrophobic face of the active site (ref 2 and 3). By analogy to the known structure of SB-benzylamide in the active site, we predict that the terminal amino acids of inhibitors of the form SB-Phe-AA will be directed toward the hydrophilic side of the active site (we base this prediction on the assumption that the SB group and aromatic side-chain of SB-Phe-AA will occupy roughly the same locations as determined for SB-benzylamide). In particular, molecular modeling predicts that SB-Phe-Gly, SB-Phe-Asn, and SB-Phe-Asp should be in close enough proximity to Asn 63, Asn 68 and Gln 93 in the CA active site to form hydrogen bonds with some or all of these amino acids. We synthesized these inhibitors and tested them for binding. Despite the fact that the polar groups should be capable of forming hydrogen bonds with polar groups on the hydrophilic side of the active site, the more polar side-chains led to decreased binding ability.

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