ORGANIC LETTERS

2000
Vol. 2, No. 9
1189−1192

Linear Free Energy Relationships Implicate Three Modes of Binding for Fluoroaromatic Inhibitors to a Mutant of Carbonic Anhydrase II

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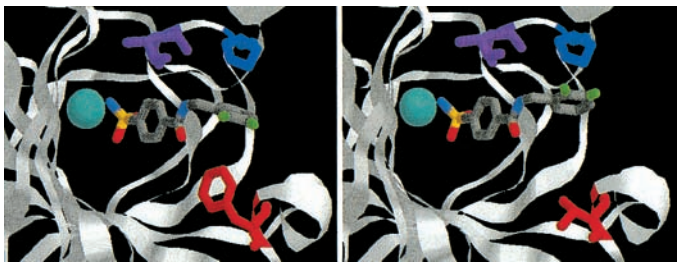
Received February 1, 2000

ABSTRACT



Linear free energy relationships between binding affinity and hydrophobicity for a library of fluoroaromatic inhibitors of F131V carbonic anhydrase II (CA) implicate three modes of interaction. X-ray crystal structures suggest that F131 interacts with fluoroaromatic inhibitors, while P202, on the opposite side of the active site cleft, serves as the site of the hydrophobic contact in the case of the F131V mutant. 2-Fluorinated compounds bind more tightly, perhaps due to the field effect of the nearby fluorine on the acidity of the amide proton.

Inhibitors of carbonic anhydrase II (CA) are composed of a sulfonamide moiety which binds to a zinc ion at the center of a deep hydrophobic cavity.[1] To prepare tight binding inhibitors, secondary interactions, including hydrophobic, aromatic, and F·H contacts, have been implicated.[1−4] Site-directed mutagenesis involving hydrophobic active site residues has also been used to elucidate interactions between small molecules and the active site of CA.[5−7]

We have recently reported that a small library of fluoroaromatic inhibitors binds to bovine CA in a manner dependent on both hydrophobicity and aromatic contacts.[2] In the present work, we have determined the affinity of this library of inhibitors for a recombinant human CA mutant where a key aromatic residue in the active site (F131) has been replaced by an aliphatic group (V). This substitution removes any possible aromatic contact between the fluoroaromatic ring of the inhibitor and CA, thereby revealing the role of hydrophobicity in the binding affinity of these inhibitors. Our results suggest, however, that additional forces still affect binding.

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(1) Jain, A.; Alexander, R. S.; Christianson, D. W.; Whitesides, G. M. *J. Med. Chem.* **1994**, *37*, 2100−2105.
(2) Doyon, J. B.; Jain, A. *Org. Lett.* **1999**, *1*(2), 183−186.
(3) DerHovanessian, A.; Doyon, J. B.; Jain, A.; Rablen, P. R.; Sapse, A. M. *Org. Lett.* **1999**, *1*(9), 1359−1362.
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(5) Nair, S. K.; Krebs, J. F.; Christianson, D. W.; Fierke, C. A. *Biochemistry* **1995**, *34*, 3981−3989.
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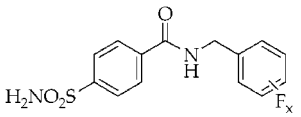
10.1021/ol005608r CCC: $19.00
Published on Web 04/06/2000

The 18 compounds that comprise our library have the general structure shown below. Dissociation constants from



wild-type (wt) and mutant proteins were determined for each of the inhibitors using a competitive binding assay.[8] Fluorescence measurements were made using a Molecular Devices SPECTRAmax Gemini microplate spectrofluorometer.

Hydrophobicity was determined as log *P*, using microemulsion electrokinetic chromatography (MEEKC).[9,10] This method takes advantage of the change in electrophoretic mobility of a molecule due to its partitioning between solution and the interior of a micelle. More hydrophobic molecules prefer the cavity of the micelle, giving them a greater net charge. On the basis of a standard curve derived from molecules having known octanol−water partition coefficients, the hydrophobicity of unknowns was conveniently determined by this method.

Dissociation constants ($K_d = 1/K$) for inhibitors dissociating from recombinant human wt and F131V CA and measured log *P* values are shown in Table 1. A linear free energy relationship (LFER) between log *K* and log *P* is shown in Figure 1 for F131V CA. Wild-type human protein showed no correlation ($R = 0.29$) between experimentally measured log *P* values and binding affinity, consistent with

Table 1. Dissociation Constants and log *P* Values for Fluorinated Inhibitors of Carbonic Anhydrase

fluorine substitution pattern	K_d* (nM)		log *P**
	F131V	wild-type	
2	0.36	2.3	2.00
2,3	0.29	1.6	2.32
2,5	0.34	2.2	2.14
2,6	0.91	3.9	1.92
2,3,6	1.3	2.4	2.01
2,3,5,6	1.8	2.2	2.22
perhydro	2.1	5.6	1.93
3	0.76	2.9	2.23
4	0.54	3.3	2.29
2,4	0.66	3.3	2.30
2,4,5	1.1	2.3	2.51
2,4,6	2.2	3.9	2.13
2,3,4,5,6	1.5	2.0	2.54
3,4	1.2	4.3	2.79
3,5	3.3	2.0	2.73
2,3,4	1.8	3.8	2.51
3,4,5	1.1	3.9	2.87
2,3,4,5	1.5	2.6	2.68

*K_d values have uncertainties of ±10%, estimated from the errors in fits of data from multiple titrations with each inhibitor and from the variation in the K_ds obtained from separate experiments. log *P* values have uncertainties of ±3%, based on separate measurements.

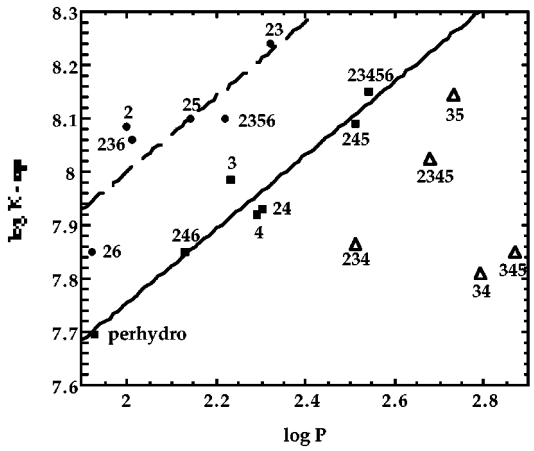


Figure 1. LFER for binding of fluorinated inhibitors to F131V carbonic anhydrase II. Filled squares, filled circles, and open triangles represent the three groups of inhibitors described in the text. The correction of log *K* included on the *y*-axis accounts for the inductive effect of a 4-benzamide substituent (ref 13).

the idea that interactions between the aromatic rings of our inhibitors and F131 of CA[3] may counter the intrinsic dependence of binding on hydrophobicity.[2,11] The LFER for the F131V mutant, however, suggests three distinct groups of inhibitors whose binding affinity is related to hydrophobicity. The tightest-binding group consists of six molecules that are fluorinated at the 2 position but not at the 4 position.[12] Five inhibitors that are minimally fluorinated at the 3,4- or 3,5-position comprise a weakly binding group whose affinity shows no correlation with hydrophobicity ($R = 0.01$). The remaining seven inhibitors form a group with intermediate affinity that does correlate with hydrophobicity and with data from Hansch and co-workers[13] (vide infra).

The two high-affinity groups of inhibitors share a common dependence on hydrophobicity (in Figure 1, $m = 0.70 \pm 0.21$, $R = 0.85$; $m = 0.69 \pm 0.08$, $R = 0.97$). The LFER for the each of these groups agrees remarkably well with respect

(8) Chen, R. F.; Kernohan, J. C. *J. Biol. Chem.* **1967**, *242*, 7813−7823. For wild-type CA, we measured $K_{d,DNSA} = 0.60\ \mu$M; for F131V CA, $K_{d,DNSA} = 21$ nM, in 20 mM KH_2PO_4 (pH 8).

(9) Ishihama, Y.; Oda, Y.; Uchikama, K.; Asakawa, N. *Anal. Chem.* **1995**, *67*, 1588−1595.

(10) A 10 mM solution of each of the fluorinated inhibitors in a microemulsion consisting of 1.66% SDS (w/v), 6.49% 1-butanol, and 0.89% heptane in 0.05 M KH_2PO_4/0.1 M H_3BO_3 (pH 7.0) electrophoresis buffer was analyzed using a Waters Quanta 4000E capillary electrophoresis system. The following parameters were used to determine hydrophobicities: capillary length = 37 cm (30 cm to the detector), 13.5 kV, detection at 214 nm.

(11) We noted an interesting trend, however, between the affinities of our library for wt and mutant protein, after normalizing each set of data for the affinity of that protein to the perhydro derivative. Inhibitors having two or fewer fluorines bind more tightly to wt CA, while larger inhibitors prefer the F131V mutant. The only exception to this rule was the 3,4,5-trifluoro derivative, which may project its fluorine substituents out into the larger portion of the conical cleft of CA, avoiding steric clashes with the bulkier active site of the wt protein.

(12) The fact that the 2,6-difluorobenzyl-derived CAI binds less tightly than is expected cannot be readily explained by any anomalies in the crystal structure of this inhibitor bound to F131V CA.

(13) Hansch, C. W.; McClarin, J.; Klein, T.; Langridge, R. *Mol. Pharmacol.* **1985**, *27*, 493−498.

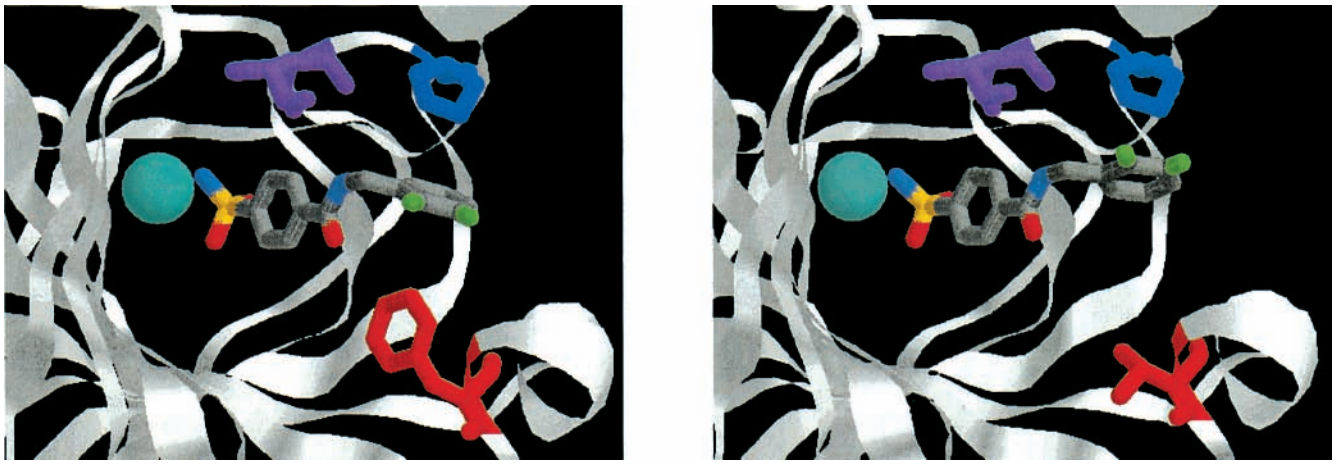


Figure 2. X-ray crystal structures of (a, left) wild-type CA and (b, right) F131V CA bound to 2,3-difluorobenzylamide-derived inhibitor. The divalent zinc ion is shown in cyan, F131 and V131 are red, T200 is purple, and P202 is blue.

to the dependence of log *K* on hydrophobicity with results from Hansch and co-workers ($m = 0.64 \pm 0.09$).[13] Hansch analyzed data for the binding of *wild-type CA*, which includes the aromatic F131 residue, to *aliphatic* inhibitors. Our results in Figure 1 represent binding of the *F131V mutant*, a protein bearing an aliphatic residue at position 131, to *fluoroaromatic* inhibitors. Although the aliphatic and aromatic partners in the interaction are inverted in our work and Hansch's, the dependence of affinity on hydrophobicity is within error. In this system, at least, aromatic/aliphatic hydrophobic contacts do not depend on the location of the aromatic or aliphatic groups.

The specific hydrophobic contacts that we have observed for fluoroaromatic inhibitors bound to wt and F131V CA are exemplified in the crystal structures shown in Figure 2. The 2,3-difluorobenzylamide-derived CAI binds to wt CA in a manner that allows contact with F131 (the *m*-hydrogen of F131 is 2.97 Å from the centroid of the fluorobenzyl ring). On the other hand, the absence of the relatively large hydrophobic surface of F131 in the case of the mutant causes the fluoroaromatic moiety to move 1.4 Å toward the aliphatic ring of P202 on the opposite side of the active site cleft.[14]

An additional interaction must account for the difference in the *y*-intercepts of the LFERs for the two families of inhibitors discussed above. Each of the inhibitors in the tighter binding group bears fluorine at the 2 position. Fluorination at the 2 position should increase the acidity of the benzylamide proton via an inductive effect. This amide proton, as well as the fluorine, can hydrogen bond to a water molecule bound to T200 in the active site.[15] Addition of fluorine at the 4 position increases the local dipole of the fluoroaromatic ring, which should decrease the strength of its interaction with the hydrophobic P202 residue. The five inhibitors that are 3,4- or 3,5-fluorinated should also have a large local dipole, weakening their interaction with P202, as indicated by their behavior in Figure 1.

Our studies of the binding of a small library of fluorinated arylsulfonamides to F131V CA have confirmed that hydrophobicity is a key determinant of the affinity of these inhibitors for the protein, with a dependence similar to that previously observed by Hansch and co-workers.[13] Secondary interactions that derive from the inductive effect of fluorine at the 2 position on the hydrogen bond donor capability of the amide proton seem to contribute to enhanced binding of 2-fluorinated inhibitors to F131V CA. To obtain support for the contacts that we have implicated, NMR measurements of NOEs[16] from $^{19}F \rightarrow {}^{1}H$ are underway, with wt and mutant protein.[17] We believe that our data help to illustrate the relevance of the modes of interaction of hydrophobic inhibitors with CA described here and will more generally provide a new paradigm relevant to the design of fluorinated drugs.

Acknowledgment. Swarthmore College, the Howard Hughes Medical Institute (R.D.M.), Pfizer (J.B.D.), the donors of the Petroleum Research Fund, administered by the

(14) The distances between the *m*-carbon of the of the fluorobenzyl ring and the *β*-carbon of P202 are 7.15 and 5.80 Å for the wt and mutant, respectively. Similar changes have been observed in crystal structures with five other fluoroaromatic CAIs bound to F131V CA: Kim, C.-Y.; Doyon, J. B.; Baird, T. A.; Fierke, C. A.; Jain, A.; Christianson, D. W., manuscript in preparation.

(15) The oxygen of the bound water (O_w) is 2.81 Å from the amide nitrogen of the inhibitor and 3.03 Å from the oxygen of T200 (O_{200}), in the case of the complex with the 2-fluorinated inhibitor. The $\angle NO_wO_{200}$ is 118°. The 2-fluorine is 4.39 Å from O_w (ref 14).

(16) Dugad, L. B.; Cooley, C. R.; Gerig, J. T. *Biochemistry* **1989**, *28*, 3955−3960.

(17) These NOE measurements may also rule out the presence of F·H contacts in solution between the inhibitors and backbone amide protons. By examining a crystal structure of the 2,3-derivative bound to wild-type CA, we noted that two N−H groups (residues 131 and 132) could form F·H contacts with bound inhibitor, given a modest conformational change. Such a conformational change in the case of the F131V protein might bring both of these N−H groups within contact distance for fluorines at the *ortho* and *meta* positions (a restricted MM2 minimization starting from the geometry found in the crystal structure of the 2,3-derivative bound to wt CA, with F131 replaced by V, in MacroModel 6.0 (Schrodinger, Inc.) allowed amino acids 124−138 to undergo the conformational change required for the F·H contacts).

American Chemical Society (A.J.), and NIH Grant GM45614 (D.W.C.) provided financial support. The SPECTRAmax Gemini Microplate Fluorometer was on loan from Molecular Devices.

Supporting Information Available: (1) ^{1}H and ^{19}F NMR data for the 2,4,6-trifluorinated inhibitor, (2) standard curve used for determination of log *P* values, and (3) bar graph of log K_{wt} − log K_{mut} versus substitution pattern. This material is available free of charge via the Internet at http://pubs.acs.org.

OL005608R