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THE NATURE OF INTERACTION BETWEEN THE CARBOXYLATE OF SUBSTRATES AND THE GUANIDINIUM MOIETY OF ARG-145 IN CARBOXYPEPTIDASE A PROBED BY INHIBITORS OF THE ENZYME

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Abstract: Replacement of the α-proton of 2-benzyl-3-hydoroxypropanoic acid, a competitive inhibitor of carboxypeptidase A with a fluoro group brought about a 2-fold increase in K_i value (0.61 mM → 1.19 mM), while pK_a value decreased by 1.4 units (4.36 → 2.95), suggesting that the carboxylate of the inhibitor and that of substrates as well are hydrogen bonded to the guanidinium moiety of Arg-145 of the enzyme.

One of important binding forces involved in the formation of Michaelis complexes of carboxypeptidase A[1] (CPA) with its substrates is interactions of the C-terminal carboxylate with the guanidinium moiety of Arg-145.[2] As part of our ongoing study to develop novel enzyme inhibitor design strategies using carboxypeptidase A as a model,[3] we were interested in knowing the nature of the interactions between the carboxylate and the guanidinium moiety. This communication describes the study aimed at to shed light on the nature of the interaction between the carboxylate and Arg-145 guanidinium moiety of the enzyme using weak competitive inhibitors of the enzyme as probes.

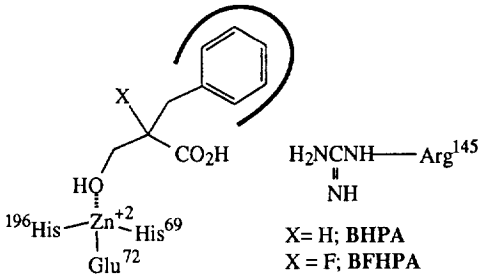
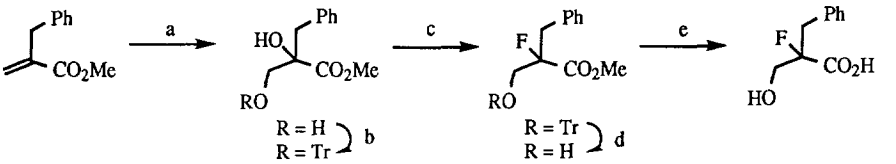


Figure 1. Schematic representation of binding mode of **BHPA** and **BFHPA**, reversible competitive inhibitors for CPA.

For the present study we have designed 2-benzyl-3-hydroxypropanoic acid (**BHPA**) and its α-fluoro derivative (**BFHPA**). They were conceived as substrate analog competitive inhibitors for the enzyme, of which the carboxylate interacts with the guanidinium base of Arg-145, the phenyl group is accommodated in the hydrophobic pocket, and the hydroxyl group coordinates to the active site zinc ion (Figure 1). The α-

fluoro group in **BFHPA** is introduced in order to augment the acidity of the carboxylate without perturbing the topological environment in the region of the α-proton: The fluoro group which is small in size has a very strong electron withdrawing power,[4] so the primary effect expected from the introduction would be lowering of p*Ka* value of the carboxylate group next to it.

BHPA was prepared as described in the literature[5] and its fluoro derivative, **BFHPA** was synthesized starting with 2-benzylacrylic acid[6] following the sequence of reactions depicted in Scheme 1,[7] and their p*Ka* values were determined[8] to be 4.36 and 2.95, respectively. The Dixon plots[9] (Figures 2 and 3) of the kinetic data obtained, under initial velocity conditions, for the hydrolysis of hippuryl-L-phenylalanine (substrate) in the presence of each inhibitor demonstrate that both inhibit the enzyme in a competitive fashion having K_i values of 0.61 and 1.19 mM for **BHPA** and **BFHPA**, respectively. We attribute the higher K_i value in the case of **BFHPA** to its lowered p*Ka* value as a consequence of the introduction of the fluoro group,[10] thus to result in weakening of the interaction of the carboxylate with the Arg-145 guanidinium group.



Scheme 1. *Reagents, conditions, and (yields)*: a) OsO_4, *N*-methylmorpholine *N*-oxide, H_2O-acetone (1:1), 36 h, (91 %); b) TrCl, TEA, DMAP (cat), 2 d, (82 %); c) diethylaminosulfur trifluoride (DAST), -78°C to rt; d) formic acid-diethyl ether (1:1), 3 h, (c and d: 52 %); e) 0.1 N LiOH in THF:MeOH:H_2O (3:1:1), overnight (66 %).

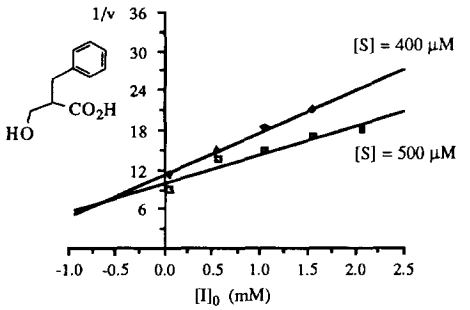


Figure 2. The Dixon plot for the inhibition of CPA-catalyzed hydrolysis of hippuryl*L*-phenylalanine by **BHPA**; 0.05 M Tris buffer, pH 7.5, 0.5 M NaCl; [E] = 368 nM at 25 °C.

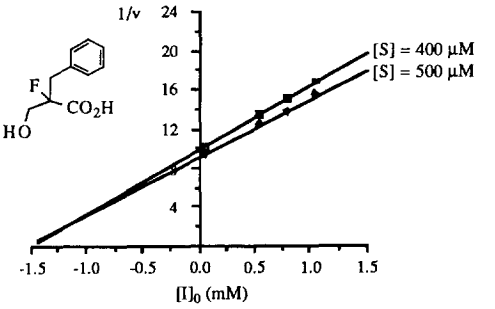


Figure 3. The Dixon plot for the inhibition of CPA-catalyzed hydrolysis of hippuryl-*L*-phenylalanine by **BFHPA**; 0.05 M Tris buffer, pH 7.5, 0.5 M NaCl; [E] = 368 nM at 25 °C.

Recently, hydrogen bonds have received renewed attention as a new type of force important in the enzymic reactions.[11] Low barrier hydrogen bond (LBHB) of remarkably high strength was claimed to contribute 10 - 20 kcal/mol of stabilization to otherwise unstable intermediates in enzyme-catalyzed reactions.[11] According

to Cleland,[11a] and Gerlt and Gassman,[11b] the difference in p*Ka* value of the two interacting moieties in the formation of a hydrogen bond governs the strength of the bond: The strength of the hydrogen bond approaches maximum as the difference (ΔpKa) in p*Ka* values becomes zero. Thus, the lowered binding affinity of **BFHPA** compared with that of the corresponding unfluorinated inhibitor can be rationalized on the basis of the LBHB, supporting that hydrogen bonds rather than electrostatic interactions are in effect between the two moieties:[12] The difference in p*Ka* value between the **BFHPA** carboxylate and the guanidinium moiety of Arg-145 is larger than that between the carboxylate of **BHPA** and the guanidinium moiety, which would presage according to the LBHB theory that **BFHPA** binds the enzyme with a lower binding affinity as observed. It is noteworthy that there is shown only 2-fold reduction in the binding affinity, whereas the p*Ka* change was amounted to 1.4 units. This small decrease in affinity suggests that the hydrogen bonds between the carboxylate and the Arg-145 guanidinium moiety are rather weak double-well hydrogen bonds.[11] The conclusion derived from the present study is consistent with the X-ray crystallographic data[13] of the CPA complex formed with a slowly hydrolyzing substrate, Gly-Tyr. Thus, distances between the heteroatoms (O, N) that are involved in the above interactions are reported to be 3.0 and 3.3 Å, which suggests that the two hydrogen bonds of unequal strength are involved. If there operates an ionic interaction, the two distances between heteroatoms are expected to be equal. A further support for the hydrogen bonds between them comes from the recent report of Melo and Ramos[14] who arrived at the conclusion from *ab initio* calculations using appropriate models that hydrogen bond in a neutral form is more stable than the ionic form in the interaction of a carboxylate with a guanidinium moiety. Figure 4 is a schematic representation of the interaction of the guanidinium moiety of Arg-145 of CPA with the carboxylate of substrates and inhibitors.

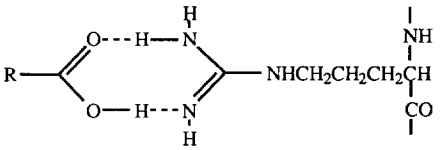


Figure 4. Proposed interaction mode of the guanidinium moiety of Arg-145 in the active site of CPA with the carboxylate of substrates and inhibitors bound to the enzyme.

In conclusion, the interaction of the carboxylate of the C-terminal amino acid residue of substrates as well as the carboxylate of substrate analogs with the guanidinium moiety of Arg-145 in binding to CPA has been usually referred as zwitterionic electrostatic interaction but the present study made with enzyme inhibitors as probes suggests that hydrogen bonds are involved as the principal binding force. Furthermore, this study demonstrates that enzyme inhibitors of a substrate analog type are usable as valuable tools for the study of the nature of weak interactions involved in binding of substrates to enzymes.

Acknowledgment: We thank the Korea Science and Engineering Foundation for the financial support of this work.

References and Notes

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7. For **BFHPA:** mp 104-105 °C; IR (KBr) cm^{-1} 3600 - 2300, 1708; ^1H NMR 300 MHz ($CDCl_3$) δ **2.96** – **3.19** (m, 2H), 3.67 – 4.00 (m, 2H), **6.97** (br, 2H), 7.21 – 7.33 (m, 5H). Anal. Calcd for $C_{10}H_{11}FO_3$: C, 60.60; H, 5.59. Found: C, 60.49; H, 5.59.
8 The p*Ka* values were measured on a Kyoto Electronics AT 400 coupled with APB 410 using 0.10 N sodium hydroxide solution as a titrant.
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10. Although the C-F bond is capable of being involved in the interaction with proton donors, they are generally weaker than the corresponding C-O and C-N groups. Being somewhat larger than hydrogen, the fluorine may be get involved in unfavorable interactions with amino acid residues in the active site. However, these steric/electronic perturbations are thought to be insignificant considering the active site structure of the enzyme elucidated by the X-ray diffractometry.
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12. We restrict our discussion to the major binding interaction of the carboxylate, that is, the interaction with the guanidinium moiety of Arg-145, although it is known that from X-ray crystal structures of CPA•Inhibitor complexes that the carboxylate is also involved in hydrogen bonding interactions with Asn-144 and Tyr-248.[1b]
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