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Modulation of the affinity of aspartic proteases by the mutated residues in active site models

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The active sites of 3 types of aspartic proteases are modeled, based on crystallographic coordinates of endothiapepsin and of a model of HIV-1 protease. The enthalpies of deprotonation from neutral to mono-anion and to dianion are calculated with semiempirical minimal neglect of differential overlap, hydrogen bonding corrected (MNDO/H). This quantum mechanical study of models for the active sites of pepsins, human renin and retroviral aspartic proteases demonstrates that the replacements of Thr-218 from pepsins by Ala in human renin and of both Ser-35 and Thr-218 by alanines in retroviral proteases increases the proton affinity and modulates the charge distribution of those active sites compared to the pepsins.

Protease, Aspartic; HIV-1; Renin; Pepsin; Model, molecular

1. INTRODUCTION

APs are a family of endopeptidases which have many common features [1] in addition to structural homology [2]. The catalytic machinery includes two closely positioned aspartic acids with highly conserved neighbor residues. Most APs are maximally active at low pH ('acid proteases'). They have diverse substrate specificities but are inhibited, to varying extents, by a common inhibitor, pepstatin A [3–5]. APs are known to be involved in a few disease conditions such as AIDS, essential hypertension, several cancers and others [6] and are now a major target for designing inhibitors.

Recent research establishes that HIV-1 protease, which processes the gag and pol polyprotein precursors towards the production of mature viruses, is an AP [7]. Like other retroviral proteases, it has a much shorter polypeptide chain than pepsins and renins, and was postulated to function in dimeric form [8]. Its crystal structure [9,10] confirms those predictions. Other divergences of HIV-1 protease are the higher pH for optimal activity [7,11] compared to pepsins, and its much weaker inhibition by pepstatin A [4,7,12] (table 1). Those characteristics are generally shared by renin [13,14].

A few pepsins have been crystallized and their reported high-resolution X-ray structures include coordinates for the heavy atoms [15–17] (C, N, O, S) as well as positions of oxygens from water molecules [18,19]. More recently, the structures of renin [20], RSV protease [21] and HIV-1 protease from two sources [9,10] have been published. Introduction of inhibitors into the crystals further reveals a remarkable rigidity of the active sites of AP except for the 'flap' region facing the active aspartates [19,22,23]. This rigidity is primarily attributed to intricate hydrogen bonding between the active site residues. Fig.1 shows the alignment of 8 active site residues which are crucial for maintaining the hydrogen-bonded structure of the catalytic centers. The role of the fourth residue of each partial sequence (which is mutated in h. renin and retroviral proteases compared to pepsins) in regulating the properties of active site aspartates has rarely been addressed and the source of optimal pH variations among the AP is poorly understood [20].

The conservation of spatial relations of those residues in the crystal structures of many native and inhibited enzymes supports the conclusion that it is possible to model the active site of an AP for which only primary structure is known, by using the published coordinates of another. We have taken this approach to study the active sites of h. renin and retroviral proteases based on the published coordinates of endothiapepsin. Models of renin [24–28] and of HIV-1 protease [29] were previously constructed for studying their interactions with inhibitors. However, some details of the structure are missing due to the lack of hydrogen coordinates in X-ray studies of proteins. Some of those proton positions are extremely important for hydrogen bonding. Correct positioning of the missing protons is energy-sensitive and requires a reliable method. Semiempirical quantum mechanical studies of H-bonding interactions have been recently improved [30]

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Abbreviations: AP, aspartic protease; MNDO/H, minimal neglect of differential overlap, hydrogen bonding corrected

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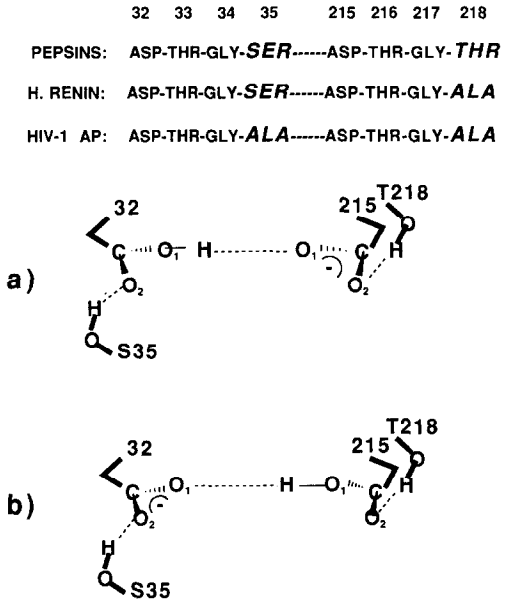

Fig.1. Active site residues of pepsins, human renin and retroviral proteases. Residue numbers are from the pepsin system. The two partial sequences of retroviral AP are from identical monomers which dimerize to form the functional enzyme. Each partial sequence of 4 residues is folded so that the terminal Ser and Thr in pepsins can form hydrogen bonds with the two aspartates. The mutations of those H-bonding residues in human renin and in retroviral proteases should affect the properties of the active site [20,34]. Two alternatives for H-bonding between the 'inner' oxygens (O_1, at ~2.9 A distance) of the aspartates in endothiapepsin are schematically drawn for the mono-anionic, presumably 'active' state. (a) Asp-32 donates an H-bond to anionic Asp-215. Due to the anionic nature of this state, Thr-218 donates an H-bond to Asp-215, and Ser-35 is a donor to the 'outer' oxygen (O_2) of Asp-32. (b) Asp-215 is the donor to Asp-32 and the two hydroxylic side chains donate H-bonds to the anionic site as in (a). Other H-bonding alternatives (with the proton on the 'outer' oxygens of the aspartates) are higher in energy. In the neutral state, the 'outer' oxygen (O_2) of Asp-215 is protonated in (a) and the H-bond from Thr-218 is lost due to proton–proton repulsion (see fig.2, upper stereo-pair). The neutral equivalent of (b) has another proton on O_2 of Asp-32 but Ser-35 remains a proton donor in the lowest energy alternative, since its position is not symmetrical to that of Thr-218. The H-bonding is strongly affected by the replacement of Thr-218 by Ala in human renin and by replacing both hydroxylic side chains with Ala in retroviral AP.

and allow us to compare alternatives for H-bonding [31] and to study the energetics of protonation/deprotonation and interactions in relatively large model systems [32].

2. MATERIALS AND METHODS

Coordinates of 8 residues from the active sites of endothiapepsin (4APE) and from the model of HIV-1 protease (1HVP), 4 from each domain, were extracted from the Brookhaven Data Bank file [33]. Hydrogens were added to the structures and their positions were optimized to reach the lowest enthalpy of formation. Only the bond lengths, but not angles, were optimized for hydrogen atoms which replace backbone carbons at the partial chain terminals (hydrogen replacing a carbonyl carbon at the N-H terminals of Asp-32 and Asp-215 as well as replacing nitrogens at the C = O terminals of each 4 residues). The H-bonding pattern was altered between the two aspartic acids both in the neutral and the mono-anionic states to study their relative stability. In both of those states, one aspartic acid or the other can preferentially contribute the hydrogen bond to bridge the oxygens. A subsequent deprotonation leads to the single dianionic state. Alternative H-bonding arrangements were tested for the aspartates and their neighbors and we report only the most stable conformations. In previous calculations [31,32] we discussed the small effect of replacing the remote atoms at partial chain terminals

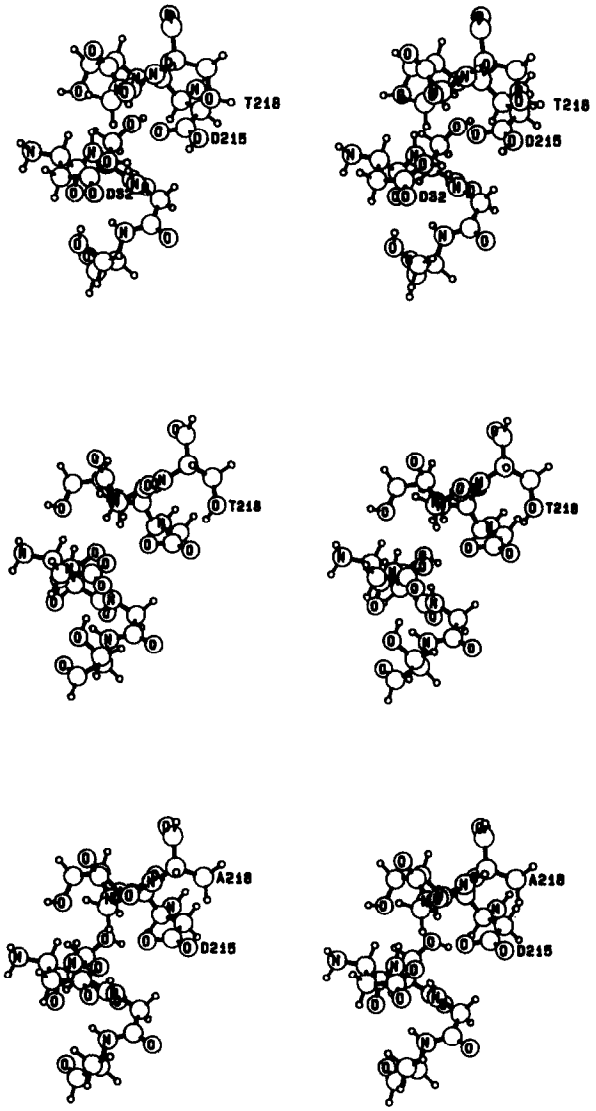

Fig.2. Stereoscopic structures of the neutral active site model of endothiapepsin (4APE, upper pair) after the addition of hydrogens and energy optimization of their positions. In this structure (corresponding to col.1, line 1 of table 2), Asp-32 is the H-bond donor, and the other proton is on the outer oxygen of Asp-215, repulsive towards the possible H-bond from Thr-218. In all the structures, the left side residues are from the N-terminal (32–35 in pepsin no.). The H-bonds in the rear are from Thr-33 (backbone N-H) to Thr-216 (side-chain oxygen) and from Thr-216 (N-H) to Thr-33 ('fireman's grip'). Threonine residues are represented without methyls on Cβ. The middle and lower pairs show, respectively, the dianionic states of pepsins and retroviral AP. The acidity of pepsin's active site is greater due to the two hydroxylic H-bonds to the aspartates, while in retroviral AP the hydroxylic side chains are mutated to alanines and lose this acidity enhancement.

Table 1

Inhibition of APs by pepstatin A and their optimal pH for substrate cleavage

	IC_{50}, 10^8 (M)	pH optimum
Pepsin	1.4	1.9–3.1
Cathepsin-D	0.9	3.1–3.3
Renin	600	6.0–7.0
AP of HIV-1	110	5.5–5.7

Values for inhibition of pepsin, cathepsin-D and renin are from [3] and their pH optima are from [14]. Inhibition of HIV-1 AP is from [7] and the pH optimum from [7,11]

on the acidity of the active site of pepsin. A reduction of the atomic presentation by transforming the two terminal NH_2 and two terminal CH=O by hydrogens does not have a qualitative effect on the relative acidities of the active site. The active site of human renin was obtained by replacing the O-H of Thr-218 by hydrogen and re-optimizing. Retroviral AP coordinates ('general retroviral AP') were obtained by a similar replacement of both hydroxyls of Thr-218 and Ser-35 from the coordinates of endothiapepsin. HIV-1 AP coordinates are from the model. The structures of pepsin's neutral active site and of the dianionic state for pepsin and retroviral AP are shown in fig.2. All calculations were conducted with MNDO/H [30], a hydrogen bonding correction to MNDO [35].

3. RESULTS AND DISCUSSION

Enthalpies of deprotonation [31] are calculated for the reactions:

$$AH_2 \rightleftharpoons H^+ + AH^- \quad (1)$$

$$AH^- \rightleftharpoons H^+ + A^{-2} \quad (2)$$

where AH_2 is the neutral active site and AH^- is the mono-ionized one. The results for total and deprotonation enthalpies are listed in table 2. Entropies were not calculated but are expected to be equal for models which are based on the same structure [31]. The calculated deprotonation enthalpies are thus a reasonable measure of the 'gas phase' acidity in the immediate vicinity of the active site residues. We have previously shown the similarity of acidities in the various pepsins and found that the alternative acidities of the aspartates (i.e. deprotonation of Asp-32 or Asp-215) are equally affected by water molecules [31]. We conclude that the calculated deprotonation enthalpies reflect intrinsic acidities which differ among the AP in this study. The proton affinity differences between pepsin and the other enzymes reflect the charge redistribution as a result of mutating the residues of pepsin, Ser-35 and Thr-218 to alanines.

From the results, we conclude that the active site of pepsins is the most acidic of the 3 due to the supportive H-bonding from Ser-35 and Thr-218. Both the active site model of HIV-1 AP, based on RSV AP coordinates and the model based on endothiapepsin ('general AP') are less acidic than pepsin, despite the large differences

Table 2

Enthalpies of formation in kcal/mol for the neutral, mono-anions and dianions in the active site models of pepsins, human renin and retroviral AP

	Pepsin	H. renin	Retroviral AP	
			General	HIV-1
1. Neutral, 32 H-bond	−524.4	−483.9	−442.2	−371.6
2. Neutral, 215 H-bond	−525.0	−482.6	−443.9	−370.2
3. Mono-anion, 32^-	−577.9	−534.7	−488.2	−424.1
4. Mono-anion, 215^-	−578.6	−531.2	−488.1	−423.5
5. Dianion	−554.6	−505.6	−457.1	−407.9
6. ΔH_1 ($\Delta\Delta H_1$)	312.1 (0.0)	314.9 (2.8)	321.4 (9.3)	313.1 (1.0)
7. ΔH_2 ($\Delta\Delta H_2$)	389.8 (0.0)	394.8 (5.0)	396.9 (7.1)	392.0 (2.2)

The results for HIV-1 AP in column 4 are for the model based on RSV AP [29]. Two alternatives exist for the neutral structures (lines 1 and 2 for each model) and for mono-anions (lines 3 and 4). ΔH_1 is the proton affinity of the more stable mono-anions (difference between the more stable of lines 3 or 4 to the more stable of lines 1 or 2) and ΔH_2 is the affinity of the dianions. They are calculated for eqns 1 and 2 as differences between products and the more stable reactants. The value of $\Delta H_f(H^+)$ is 365.7 kcal/mol [31]. $\Delta\Delta H_1$ and $\Delta\Delta H_2$ are, respectively, the differences of proton affinities for first and second ionizations with respect to pepsin: more positive values are equivalent to lower acidities

between the overall enthalpies of those two (columns 3 and 4 in table 2). In pepsins, alternative acidities of Asp-32 (difference between values on lines 1 and 3 in column 1, table 2) and Asp-215 (difference between lines 2 and 4) are nearly equal due to the approximately symmetrical hydrogen bonding arrangement in their vicinity. The same is found for the model active site of general retroviral AP and from the model of HIV-1 AP. In human renin, the asymmetric transformation of only one H-bonding residue (Thr-218) results in a preferential ionization of Asp-32. The active site first and second acidities of h. renin and of retroviral AP are thus found to be lower than those of pepsin due to less hydrogen bonding to the aspartates. This result generally correlates with the experimental pH profiles for these enzymes [7,11,14]. The source of pH variation is, however, more complex: the optimum for mouse submaxillary gland renin is ~8.0 [36] with both H-bonding residues Ser-35 and Ser-218 present, and a significantly different proportion of basic to acidic residues exists in different AP [20]. However, the experimental pH profiles for AP are not measured under equal conditions, as the substrate of each aspartic protease is different [14].

The enthalpies determined in this study are a measure of proton interactions with the active site aspartates. While a proton carries a relatively large positive charge, inhibitor atoms can be positively charged to a smaller extent which will result in corresponding but smaller differences of interaction energies with the active sites in the 3 enzymes. A general choice for designing in-

hibitors, while exploiting this property, would be neutral or positively charged molecules with proton-donating groups. Thus, in addition to the present attempts to design specific inhibitors which are peptide analogs and interact with different subsites of AP, our results support a basis for designing smaller, non-peptide novel inhibitors which can interact specifically with the immediate vicinity of AP active sites. Based on our results, we expect that hydrogen bonding by an inhibitor to the mono anionic state of the active site should be stronger in HIV-1 AP and in h. renin than in pepsins.

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REFERENCES

[1] Kostka, V. ed. (1985) Aspartic Proteases and their Inhibitors, De Gruyter, Berlin.
[2] Tang, J. and Wong, R.N.S. (1987) J. Cell. Biochem. 33, 53–63.
[3] Aoyagi, T., Yasigawa, Y., Kumagai, M., Hamada, M., Morishima, H., Takeuchi, T. and Umezawa, H. (1973) J. Antibiot., Tokyo 26, 539–541.
[4] Katoh, I., Yasunaga, T., Ikawa, Y. and Yoshinaka, Y. (1987) Nature 329, 654–656.
[5] Seelmeier, S., Schmidt, H., Turk, V. and Van der Helm, K. (1988) Proc. Natl. Acad. Sci. USA 85, 6612–6616.
[6] Kay, J., Jupp, R.A., Norey, C.G., Richards, A.D., Reid, W.A., Taggart, A.T., Sanleff, I.M. and Dunn, B.M. (1988) in: Proteases II: Potential Role in Health and Disease (Hohl, W.H. and Heidland, A. eds) pp.1–11, Plenum, New York.
[7] Darke, P.L., Leu, C.-T., Davis, L.J., Heimbach, J.C., Diehl, R.E., Hill, W.S., Dixon, R.A.F. and Sigal, I.S. (1988) J. Biol. Chem. 264, 2307–2312.
[8] Pearl, L.H. and Taylor, W.R. (1987) Nature 329, 351–354.
[9] Navia, M.A., Fitzgerald, P.M.D., McKeever, B.M., Leu, T.-C., Heimbach, J.C., Herber, W.K., Sigal, I.S., Darke, P.L. and Springer, J.P. (1989) Nature 337, 615–620.
[10] Wlodawer, A., Miller, M., Jaskolski, M., Sathyanarayana, B.K., Baldwin, E., Weber, I.T., Selk, L.M., Clawson, L., Schneider, J. and Kent, S.B.H. (1989) Science 245, 616–621.
[11] Richards, A.D., Roberts, R., Dunn, B.M., Graves, M.C. and Kay, J. (1989) FEBS Lett. 247, 113–117.
[12] Schneider, J. and Kent, S.B.H. (1988) Cell 54, 363–368.
[13] Miller, R.P., Poper, C.J., Wilson, C.W. and DeVito, E. (1972) Biochem. Pharmacol. 21, 2941–2944.
[14] Maibaum, J. and Rich, D.H. (1988) J. Med. Chem. 31, 625–629.
[15] Bott, R., Subramanian, E. and Davies, D.R. (1982) Biochemistry 21, 6856–6962.
[16] James, M.N.G. and Sielecki, A.R. (1983) J. Mol. Biol. 163, 299–361.
[17] Pearl, L. and Blundell, T.L. (1984) FEBS Lett. 174, 96–101.
[18] James, M.N.G. and Sielecki, A.R. (1985) Biochemistry 24, 3701–3713.
[19] Suguna, K., Bott, R.R., Padlan, E.A., Subramanian, E., Sheriff, S., Cohen, G.H. and Davies, D.R. (1987) J. Mol. Biol. 196, 877–900.
[20] Sielecki, A.R., Hayakawa, K., Fujinaga, M., Murphy, M.E.P., Fraser, M., Muir, A.K., Carilli, C.T., Lewicki, J.A., Baxter, J.D. and James, M.N.G. (1989) Science 243, 1346–1351.
[21] Miller, M., Jaskolski, M., Rao, J.K.M., Leis, J. and Wlodawer, A. (1989) Nature 337, 576–579.
[22] James, M.N.G., Sielecki, A.R., Salituro, F., Rich, D.H. and Hofman, T. (1982) Proc. Natl. Acad. Sci. USA 79, 6137–6141.
[23] Foundling, S.I., Cooper, J., Watson, F.E., Cleasby, A., Pearl, L.H., Sibanda, B.L., Hemmings, A., Wood, S.P., Blundell, T.L., Valler, M.J., Norey, C.G., Kay, J., Boger, J., Dunn, B.M., Leckie, B.J., Jones, D.M., Atrash, B., Hallet, A. and Szelke, M. (1987) Nature 327, 349–352.
[24] Blundell, T.L., Cooper, J., Foundling, S.I., Jones, D.M., Atrash, B. and Szelke, M. (1987) Biochemistry 26, 5584–5590.
[25] Carlson, W.D., Hardschumacher, M., Summers, N., Karplus, M. and Haber, E. (1987) J. Cardiovasc. Pharmacol. 10, s91–s93.
[26] Haber, E., Hui, K.Y., Carlson, W.D. and Bernatowicz, M.S. (1987) J. Cardiovasc. Pharmacol. 10, s54–s58.
[27] Akhane, K., Umeyama, H., Nakugawa, S., Moriguchi, I., Hirose, S., Iizuka, K. and Murakami, K. (1985) Hypertension 7, 3–12.
[28] Plattner, J.J., Marcotte, P.A., Kleinert, H.D., Stein, H.H., Greer, J., Bolis, G., Fung, A.K.L., Bopp, B.A., Luly, J.R., Sham, H.L., Kempf, D.J., Rosenberg, S.H., Dellaria, J.F., De, B., Merits, I. and Perun, T.J. (1988) J. Med. Chem. 31, 2277–2288.
[29] Weber, I.T., Miller, M., Jaskolski, M., Leis, J., Skalka, A.M and Wlodawer, A. (1989) Science 243, 928–931.
[30] Goldblum, A. (1987) J. Comp. Chem. 8, 835–849.
[31] Goldblum, A. (1988) Biochemistry 27, 1653–1658.
[32] Goldblum, A. (1988) Biochem. Biophys. Res. Commun. 159, 450–460.
[33] Bernstein, F.C., Koetzle, T.F., Williams, G.J.B., Meyer, E.F., jr, Brice, M.D., Rodgers, J.R., Kennard, O., Shimanuchi, T. and Tasumi, M. (1977) J. Mol. Biol. 112, 535–542.
[34] Carlson, W., Karplus, M. and Haber, E. (1985) Hypertension 7, 13–26.
[35] Dewar, M.J.S. and Thiel, W. (1977) J. Am. Chem. Soc. 99, 4899–4907.
[36] Ondetti, M.A. and Cushman, D.W. (1982) Annu. Rev. Biochem. 51, 283–308.