Evidence for diversity of substrate specificity among members of the chymase family of serine proteases

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Abstract The term chymase is used to signify a chymotrypsin-like protease stored within the secretory granules of mast cells. Primarily based on amino acid sequence homology, 18 chymases have been identified among different animals. This study, which compares the structure of the primary specificity pocket (S1 subsite), defines a subgroup of four chymases likely to have a substrate specificity with more elastase- than chymotrypsin-like qualities. This difference is due, primarily, to finding a Val instead of a Gly at residue 199, a position corresponding to Gly216 in bovine chymotrypsin and Val216 in neutrophil and porcine elastases. Chymases with Val at 199 are found only in animals expressing multiple chymases, consistent with the premise that their substrate specificity differs from that of chymases with Gly at 199. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Mast cell; Serine protease; Chymase; Chymotrypsin; Elastase; S1 subsite; Substrate specificity

1. Introduction

The term chymase was intended to signify the presence of a chymotrypsin-like serine protease in mast cells [1]. Primarily based on sequence homology and immunological localization, 18 chymases have been identified forming a unique sub-family of serine proteases [2,3]. Despite the number [3–10] only a few chymases have been isolated and characterized biochemically. Hydrolytic studies characterizing the substrate specificities of human, rat 1 (rat mast cell protease (RMCP)-1), rat 2 (RMCP-2), and dog chymases demonstrate a preference for aromatic residues at the P1 position of substrates, consistent with a chymotrypsin-like specificity [11–14]. Like human chymase (HC), mouse mast cell protease 4 (MMCP-4), hamster chymase 1, and the newly discovered rat vascular chymase, have been shown to be efficient converters of angiotensin I to II [7,10,15], implying their chymotrypsin-like specificity.

Using standard nomenclature [16], the P1 residue of a peptide/protein substrate forms part of the scissile bond (P1–P1' bond) and interacts with the S1 subsite of the protease. In the case of chymotrypsin/trypsin family proteases, the S1–P1 interaction typically defines the general specificity of the protease; that is, whether the protease exhibits chymotrypsin-, trypsin- elastase- or aspase-like activity [17–22]. The crystal structure of recombinant human chymase (rHC) bound to the peptide inhibitor succinyl (Suc)AAPF-chloromethylketone (CMK) defined the S1–S4 subsite structure of this protease [23]. The S1 subsite appeared as a deep slot-like cleft with a mostly hydrophobic interior that could readily accommodate an aromatic sidechain. Consistent with proteases closely related to chymotrypsin, the S1 subsite of HC was formed by three sequence segments: 176–182, 196–203, and 206–209 [23]. Based on alignment to bovine chymotrypsinogen, these residues correspond to 189–195, 213–220, 225–228 [23]. Focusing on the identity of the key residues forming the S1 subsite of all chymases, we define a distinct subgroup likely to have a substrate specificity different from that expected for a chymase. The amino acid differences distinguishing this subgroup suggest a change towards an elastase-like specificity. This is supported by characterization of rHC mutated to resemble this subset.

2. Materials and methods

2.1. Materials

Substrates were from Bachem (Philadelphia, PA, USA) and buffers were from Calbiochem (La Jolla, CA, USA). Gelcode Blue was from Pierce (Rockford, IL, USA).

2.2. Recombinant proteases and inhibitors

Variants rHC-G216V and rHC-S189N (G199V and S176N by the sequence of HC) were produced by mutation of rHC previously characterized [23]. The mutations were introduced by the overlap extension PCR method [24] and the nucleotide sequences of the mutated chymases were confirmed by DNA sequence analysis. Similar to that previously described for rHC [25], variants were expressed as the three-part pseudo-zymogen fusion protein, *N*-ubiquitin-enterokinase cleavage site-chymase-C, in a baculovirus–insect cell system. Activation by enterokinase and purification of each variant was similar to that previously described for rHC [25]. Purified variant concentrations were estimated from $A_{280\,nm}$ where $\varepsilon_{280\,nm}$ HC = 24 500 M^{-1} cm^{-1}. This value was calculated based upon three Trp, nine Tyr, and three cystine per HC molecule [26].

Serpins r(antichymotrypsin)ACT-L358 and rACT-L358W were produced in *Escherichia coli* and purified as previously described [27]. The concentration of rACT was determined by titration with standardized bovine chymotrypsin and the concentration of rACT-L358W was determined by titration at pH 5.5 with standardized rHC [28]. Titrations were performed in a solution of 1.0 M NaCl, containing either 0.1 M Tris pH 8.0, or 0.1 M MES, pH 5.5.

2.3. Biochemical methods

Hydrolysis of peptide-nitroanilide (NA) substrates was followed

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Abbreviations: ACT, antichymotrypsin; CMK, chloromethylketone; HC, human chymase; MMCP, mouse mast cell protease; NA, nitroanilide; rHC, recombinant human chymase; RMCP, rat mast cell protease; SI, stoichiometry of inhibition; Suc, succinyl

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continuously (readings taken at 1-s or 10-s intervals) by measurement of $A_{410\,nm}$ using a Beckman DU640 spectrophotometer. Kinetic parameters were determined from initial velocity vs. substrate concentration plots, assuming $\varepsilon_{410\,nm}$ NA = 8800 M^{-1} s^{-1}. Assay conditions were 1.0 M NaCl, 0.1 M Tris–HCl pH 8.0, 9% Me_2SO, 25°C. Initial velocities were determined over a 3-min assay period for rHC-S189N, and over a 30-min assay period for rHC-G216V. Enzyme concentrations in both cases were such that substrate consumption did not exceed 5% of total, and plots of product accumulation vs. time were linear. Data were analyzed using the curve fitting programs of Igor Pro from Wavemetrics. Reactions of rHC with serpins were performed in a solution of 1.0 M NaCl, 0.1 M Tris pH 8.0, 25°C. Resolution of reactant/products of chymase–serpin reactions by SDS–PAGE was performed using the gel compositions of Laemmli [29]; the separating gel consisted of 12% acrylamide/1% bis-acrylamide. Protein bands were visualized by staining with Gelcode Blue.

2.4. Computer modeling

Swiss-pdbViewer from Glaxo-Wellcome was used to mutate the rHC-peptide CMK crystal structure [23]; accession number 1PJP. Potential steric clashes were evaluated over a range of rotomer structures according to the mutation program within the Swiss-pdbViewer software.

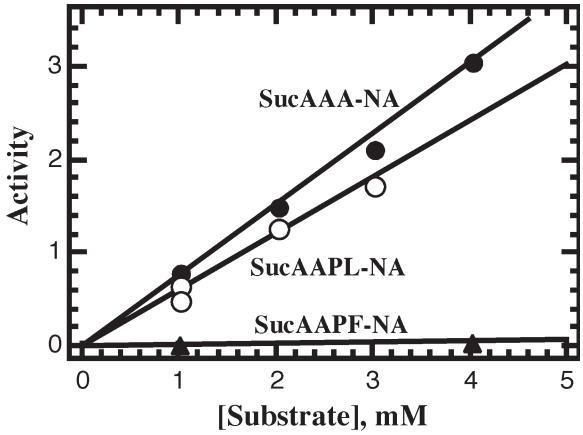

Fig. 1. Hydrolysis of peptide-NA substrates by rHC-G216V. Activity is plotted as $\Delta A_{410\,nm}$/(min × mM protease). In actual measurements the rHC-G216V concentration was 1 μM. Product accumulation to obtain activity was monitored for 30 min. Lines through data were calculated using linear least squares analysis.

3. Results

3.1. Structure of the S1 subsite among chymase family members

The amino acid sequences of the residues forming the S1 subsite of 18 chymases are compared in Table 1. Chymotrypsinogen numbering assigned to crystallized HC [23] is used to reference residues; this numbering system denotes structural location and/or functional homologies among different members of the chymotrypsin/trypsin family. Important positions for determining the S1 subsite specificity of chymotrypsin-, trypsin-, and aspase-like proteases are residues 189 and 226 at the bottom of the S1 pocket [17–22]. More important for determining elastase-like specificity is position 216 located at the mouth of S1 pocket. In elastases position 216 is usually a Val residue while in other serine class proteases this position is highly restricted to a Gly residue [17,30,31].

As shown in Tables 1 and 2, chymases exhibit a number of different residues at positions 189. However, none are charged residues found in trypsin- and aspase- like proteases. Examination of position 216 reveals that 4 of the 18 chymases have Val instead of Gly at position 216 (Table 1, marked in bold type). Further inspection finds additional differences highly specific for Val216 chymases (Table 2). All exhibit Asn, Val, and Ala at residue positions 189, 190, and 213, respectively. The constancy of this pattern suggests that Val216 chymases define a specific subgroup. The arrangement of Val190, Ala213, and Val216 also is found in the S1 subsite of human neutrophil elastase (Table 2).

Table 1
The primary structure of the S1 subsite of chymases

Chymase[a]	Residues 189–195[b] (176–182)	213–220 (196–203)	225–228 (206–209)
Human	S-A-F-K-G-D-S	V-S-Y-G-R-S-D-A	P-A-V-F
Rat 1	S-A-Y-K-G-D-S	V-S-Y-G-R-G-D-A	P-A-V-F
Rat 2	A-A-F-M-G-D-S	V-S-Y-G-H-P-D-A	P-A-I-F
Rat 3	L-T-Y-K-G-D-S	V-S-H-G-R-G-R-G	P-I-I-F
Rat 4	L-A-Y-K-G-D-S	V-S-H-G-P-D-R-G	P-I-I-F
Rat 5	N-V-Y-K-G-D-S	A-S-Y-**V**-H-P-N-A	P-A-V-F
Rat VCH	T-A-Y-T-G-D-S	V-S-Y-G-H-P-D-S	P-A-V-F
Baboon	S-A-F-K-G-D-S	V-S-Y-G-R-L-D-A	P-A-V-F
Dog	S-A-F-K-G-D-S	V-S-Y-G-Q-N-D-A	P-A-V-F
Mouse 1	T-A-Y-M-G-D-S	V-S-Y-G-D-S-H-G	P-A-V-F
Mouse 2	S-I-G-Q-G-D-S	A-S-------S-Y-E	P-A-V-F
Mouse 4	S-A-Y-K-G-D-S	V-S-Y-G-R-G-D-A	P-A-V-F
Mouse 5	N-V-Y-K-G-D-S	A-S-Y-**V**-H-R-Q-A	P-A-V-F
Mouse 9	S-V-Y-M-G-D-S	V-S-S-G-R-G-N-A	P-A-I-F
Hamster 1	S-A-Y-K-G-D-S	V-S-Y-G-R-G-D-A	P-A-V-F
Hamster 2	N-V-Y-K-G-D-S	A-S-Y-**V**-L-R-N-A	P-S-V-F
Gerbil 1	S-A-Y-K-G-D-S	A-S-Y-G-R-G-D-A	P-A-V-F
Gerbil 2	N-V-Y-K-G-D-S	A-S-Y-**V**-R-R-N-A	P-A-V-F

[a]Human, baboon, dog, rat 1 and 2 chymases, and cathepsin G sequences are from Chandrasekharan et al. [3]. Rat 3–5 chymase sequences are from Lützelschwab et al. [9], and the rat vascular chymase sequence (VCH) is from Guo et al. [10]. The primary structure of rat chymase 5 is identical to that termed rat chymase 3 by Ide et al. [42]. Mouse chymases 1, 2, 4, 5, and 9 sequences are from Sali et al. [4] and Hunt et al. [6]. Hamster chymase 1 and 2 are from Shiota et al. [7] and Shiota et al. [8], and gerbil chymase sequences are from Itoh et al. [5].
[b]Numbers without parentheses are based on homology to chymotrypsin and numbers in parentheses are chymase sequence numbers.

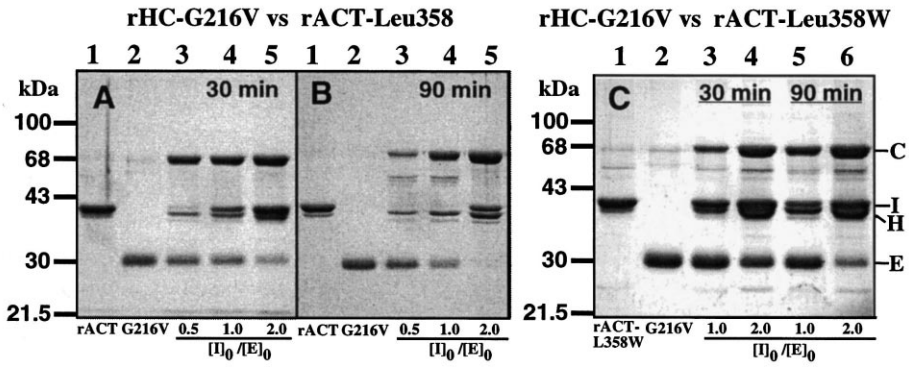

Fig. 2. SDS–PAGE analysis of rHC-G216V interaction with rACT-L358 (A, B) and rACT-L358W (C). Inhibitor concentration, $[I]_0$, was varied to achieve the reaction stoichiometries reported below lanes 3–5 in (A, B) and 3–6 in (C). The protease concentration, $[E]_0$, was held constant at 3.0 μM. $[E]_0$ and $[I]_0$ in control lanes was 3.0 μM (75 and 135 μg/ml, respectively). Reactions were performed in a total volume of 25 μl and incubations were for 30 or 90 min at 25°C. They were stopped by rapid freezing in liquid nitrogen. Immediately after thawing, reactions were denatured by dilution to 60 μl with hot SDS–DTT and further heating at 90°C for 10 min. The entire sample was resolved by SDS–PAGE. Letters on the right side of the gel signify the migration of the protease–serpin complex (C), intact inhibitor (I), cleaved inhibitor (H), and free enzyme (E). Cleavage at the reactive site of rACTs results in fragmentation of the protein into two bands of size approximately 40 kDa and 4 kDa. The 4-kDa fragment is not resolvable on the separation gel used in these studies.

3.2. Catalytic properties of rHC variants rHC-S189N and rHC-G216V

To date a Val216 chymase has not been isolated. The ability to express HC in recombinant form allows for mutation of its S1 subsite to evaluate the role of specific residues on substrate hydrolysis. Given the distinctiveness of the residues found at positions 189 and 216 of Val216 chymases, two rHC variants, rHC-S189N and rHC-G216V, were produced and characterized. rHC-S189N demonstrated a substrate specificity similar to that of native HC and rHC. That is, it hydrolyzed the substrate SucAAPF-NA more efficiently than SucAAPL-NA. Values for k_{cat}/K_M, k_{cat} and K_M were 9.2×10^4 M^{-1} s^{-1}, 55 s^{-1} and 0.6 mM for SucAAPF-NA and 2×10^3 M^{-1} s^{-1}, 3 s^{-1}, and 1.7 mM for SucAAPL-NA. The catalytic activity of rHC-S189N was titratable by the chymase inhibitor, rACT-L358W [28]. Similar to HC, the stoichiometry of inhibition (SI) obtained for this measurement was near 1. The stoichiometric titration of rHC-S189N activity by rACT-L358W confirms the concentration of the protease estimated from $A_{280\,nm}$.

In contrast, rHC-G216V demonstrated catalytic properties unique for a chymase. As shown in Fig. 1, the variant at 1 μM concentration did not catalyze the hydrolysis of the substrate SucAAPF-NA measured by NA production at $A_{410\,nm}$. Under the same assay conditions and 1 mM substrate, 4 nM HC would produce a readily measurable hydrolytic rate of 0.10 $\Delta A_{410\,nm}$/min. Also evaluated were four additional substrates with different P1 residues. Catalytic activity was observed for SucAAA-NA and SucAAPL-NA (Fig. 1), but not detected for SucAAPV-NA and SucAAPM-NA (not shown). k_{cat}/K_M values for SucAAA-NA and SucAAPL-NA hydrolysis calculated from the slopes of lines fit to the data in Fig. 1 were 1.4 M^{-1} s^{-1} and 1.2 M^{-1} s^{-1}. The hydrolytic efficiencies of both substrates are uncharacteristically low for a true peptide-NA substrate. k_{cat}/K_M values for the hydrolysis of SucAAA-NA by neutrophil and porcine pancreatic elastases are 570 M^{-1} s^{-1} and 6300 M^{-1} s^{-1}, respectively [32]. Proteinase 3, a second elastase from human neutrophils, does not hydrolyze SucAAA-NA, but does hydrolyze MeO-SucAAPV-NA exhibiting a k_{cat}/K_M value of 3000 M^{-1} s^{-1} [33].

Computer modeling studies replacing residues Ser189 by Asn and Gly216 by Val in the chymase–peptide CMK crystal structure are supportive, in part, of the hydrolytic observations. The isopropyl sidechain of Val216 was found to project

Table 2
S1 subsite structure of V216 and G216 chymases and neutrophil elastase

Protease	Residues 189–195	213–220	225-228
V216 Chymase[a]	N-V-Y-K-G-D-S	A-S-Y-V-H-R-N-A L P R	P-A-V-F S
Gly216 chymases[b]	S-A-Y-K-G-D-S A V F M T L	V-S-Y-G-R-G-D-A A H H S R G S P P H Q D N D L N	P-A-V-F I I S
Neutrophil Elastase	G-V-C-F-G-D-S	A-S-F-V-R-G-G-C	P-D-A-F

[a]Most common residue at each position is reported in the top row of Gly216 and Val216 chymases.
[b]Of the Gly216 chymases, Val190 is only found in mouse chymase 9 and Ala213 is only found in gerbil chymase 1. The residues of mouse chymase 2 were omitted due to the unusual structure of its S1 subsite which includes a three-residue deletion.

into the S1 subsite and clash with the buried benzyl group of the P1 phenylalanyl residue. Steric clashes with the buried phenylalanyl residue were not observed with Asn at 189.

3.3. Characterization of the interaction of rHC-G216V with serpins rACT-L358 (wild type-ACT) and rACT-L358W

To further evaluate the substrate specificity of rHC-G216V and to eliminate the possibility of a contaminating protease in small amounts, the inhibition of rHC-G216V with two serpins was evaluated. Inhibition by serpins is typically reflective of the catalytic competence and the substrate specificity of a protease. Serpins inhibit proteases by trapping them in a 1:1 covalent complex. The complex is a distorted form of the serpin acyl–protease complex produced as the protease attempts to hydrolyze the reactive site peptide bond (P1–P1') of the serpin [34]. The inhibition mechanism has been likened to that of a suicide substrate [35]. Consistent with suicide substrates, complex formation is not always 100% efficient and some cleaved serpin is found generated concurrent with inhibition. Cleaved serpin is not inhibitory and its generation results in SI values greater than 1 [14,36]. HC-serpin reactions are an prime example of these reaction properties. Inhibition by rACT-L358 and the reactive site variant rACT-L358W demonstrate SI values of 4 and 1.5 and kinh values of 2×10^4 M^{-1} s^{-1} and 2×10^5 M^{-1} s^{-1}, respectively [14].

As shown by SDS–PAGE in Fig. 2, the reaction of rHC-G216V with both rACT-L358 and rACT-L358W results in the production of a high molecular weight band (C) indicative of the covalent protease–serpin complex. Analyses demonstrate the time dependence of complex formation at different reaction stoichiometries. The complete disappearance of the protease band (E) by 90 min in the rACT-L358 reaction confirms that rHC-G216V is a catalytically competent protease and that all the protease in the preparation was active. Complexes were stable as demonstrated by a similar analysis of identical reactions allowed to proceed for 18–72 h (not shown). Cleaved inhibitor also was generated as demonstrated by the appearance of a band (H) immediately below that of intact inhibitor. The proportion of cleaved inhibitor to serpin–chymase complex estimated from the relative intensities of the bands suggests that the SI value for the reaction of rHC-G216V with both serpins is between 1 and 2.

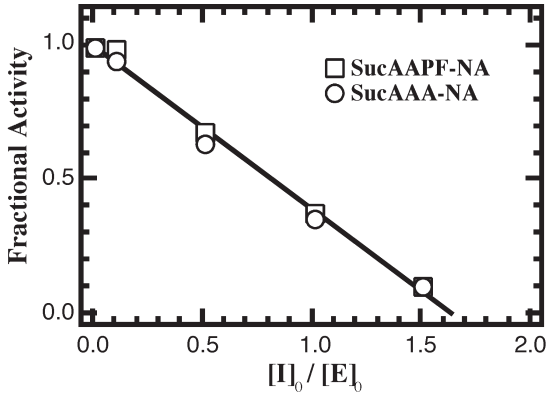

Fig. 3. Titration of rHC-G216V hydrolytic activity by rACT. Reactions containing 25 μM enzyme were incubated overnight at 25°C with increasing amounts of rACT. To assay for residual activity, reactions were diluted 10-fold with assay media containing 4.5 μM SucAAA-NA or 3 μM SucAAPL-NA, and product accumulation was monitored for 30 min. Residual activities were determined from the rate of product accumulation and fractional activities were estimated using the control activity (no inhibitor for incubation time) as unity.

rACT titration of rHC-G216V hydrolytic activity is shown in Fig. 3. The hydrolysis of both SucAAA-NA and SucAAPL-NA was inhibited in a manner that required a near stoichiometric amount of inhibitor to enzyme. The SI value established from the titration was between 1 and 2. An SI >1 is consistent with SDS–PAGE analyses of reactions (Fig. 2) showing two reaction products: protease–inhibitor complex and some cleaved inhibitor. These results demonstrate that the turnover of both substrates is due to catalysis by rHC-G216V.

The reaction of rACT-G216V with rACT-L358 and rACT-L358W is reflective of an altered substrate specificity. Based on the disappearance of the protease band in the gel analyses shown in Fig. 2, rACT-L358 appears as a somewhat more efficient inhibitor of rHC-G216V than rACT-L358W. This finding is consistent with a preference for Leu > Trp at the P1 position of rACT and is in contrast to that observed for HC (see above). Inhibition of rACT-G216V by rACT-L358W was extremely slow; no more than half complete by 30 min as shown in Fig. 2. Assuming that the reaction conditions in Fig. 2 approximate second order conditions ([E] = [I] = 3 μM), inhibition of HC (kinh = 2×10^5 M^{-1} s^{-1}) would have been half complete by approximately 2.0 s.

4. Discussion

Based on the distinctiveness of residues forming S1 subsite, a subgroup of chymases was identified that may exhibit a substrate specificity different from that expected of proteases classified as members of the chymase family [3–10]. Most distinctive was Val instead of Gly at position 216 and Asn instead Ser at position 189 (Table 2). Residues at these positions are known to influence the S1 subsite specificity of serine proteases [17–22]. Characterization of two mutants of rHC, one with Ser189 (176 chymase numbering) changed to Asn and the other with Gly216 (199 chymase numbering) changed to Val, demonstrated that a Val at position 216 could have a marked effect on substrate specificity. rHC-G216V did not hydrolyze the common chymotrypsin/chymase substrate Suc-AAPF-NA, but did hydrolyze peptide-NA substrates with Ala and Leu residues at the P1 position. The tendency to hydrolyze substrates with Ala and Leu instead of Phe at the P1 position suggests that the substrate specificity of Val216 chymases may be more elastase-like than chymotrypsin-like [11,17,30,32,33,37,38].

Val instead of Gly at position 216 is characteristic of human neutrophil elastase, proteinase 3 and porcine pancreatic elastase [30,31]. In elastases, the isopropyl sidechain of Val216 functions to constrict the interior the S1 pocket [17,30], limiting its specificity to relatively small aliphatic residues [11,32,33,37,38]. The computer model of mouse chymase 5 (MMCP-5 obtained from the web site of A. Sali, http://tatiana.rockefeller.edu/), an example of a Val216 chymase, shows the isopropyl sidechain of Val216 protruding into the S1 pocket opening, in a manner similar to elastases [4]. Modeling of Val at position 216 in the chymase–peptide inhibitor crystal structure also shows the isopropyl sidechain protruding into the S1 pocket and clashing with the buried benzyl group of the P1–Phe residue of the inhibitor. The catalytic properties of

rHC-G216V were not strictly elastase-like, however. rHC-G216V did not hydrolyze peptide-NA substrates with Met or Val at P1 and was inhibited, albeit at a slow rate, by an rACT variant with a Trp residue at the P1 position.

HC is the first chymotrypsin-like protease to have Gly216 replaced by another residue. Three trypsin-like proteases have been described with a residue other than Gly at the 216 position: (1) A rat trypsin variant with Gly216 mutated to Ala was produced to study the role of this substitution on substrate specificity [39]; (2) a protein C variant with Gly mutated to Ser was discovered as the underlying cause of a hemolytic disorder [40]; and (3) a human tryptase isozyme, termed α-tryptase, has Asp instead of Gly at position 216 [41]. Similar to that observed for rHC-G216V, characterization of these trypsin-like proteases demonstrated greatly reduced or non-detectable catalytic activity for substrates hydrolyzed by their Gly216 counterparts.

The low catalytic efficiency of rHC-G216V for model substrates SucAAA-NA and SucAAPL-NA questions the capability of Va1216 chymases to function as effective elastases. rHC-G216V may not reflect fully the catalytic potential of Val216 chymases. Val216 chymases exhibit additional residue changes in the S1 pocket that are highly conserved. These changes, Ala190 to Val and Val213 to Ala, make the S1 pocket of Val216 chymases even more similar to neutrophil elastase (Table 2). Although less likely, the current study does not eliminate the possibility that amino acid differences between Gly216 and Val216 chymases away from the S1 pocket somehow compensate for Val216, and that a true Val216 chymase will demonstrate chymotrypsin-like specificity similar to Gly216 chymases. To define the substrate specificity of Val216 chymases with more certainty, a genuine member of this subgroup needs to be isolated and studied biochemically.

At least four different animals, rats, mice, gerbils and hamsters, express a Val216 chymase (Table 1). Each animal expressing a Val216 chymase also expresses at least one Gly216 chymase. Thus, the presence of a Val216 chymase appears to be in addition to a chymase with chymotrypsin-like specificity. This observation is consistent with the premise that Val216 and Gly216 chymases have different biological functions. Phylogenetic trees developed from primary structure comparisons have subdivided the chymase family into two major groups termed α and β [3,15]. This division has been related functionally to the ability of α chymases to convert efficiently angiotensin I to II [3]. Recent evidence disputes the restriction of angiotensin conversion to α chymases [10,15]. The current study further questions the functional significance of the α and β subgrouping as phylogenetic trees show Val216 chymases within the α chymase subgroup [3,15]. Val216 chymases likely would not be efficient angiotensin converters because recognition of a P1–Phe is required. Interestingly, Val216 chymases are placed as a subdivision or branch within the α chymase subgroup, an observation perhaps consistent the functional distinction of these chymases proposed in this study.

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