The Role of Methionine-192 of the Chymotrypsin Active Site in the Binding and Catalysis of Mono(amino acid) and Peptide Substrates†

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ABSTRACT: We find that specific oxidation of the Met-192 residue in δ-chymotrypsin to methionine sulfoxide results in a twofold increase in $K_{m(app)}$ and unchanged k_{cat} in the hydrolysis of *N*-acetyl mono(amino acid) amide substrates. However, the catalyzed hydrolyses of *N*-acetyl dipeptide amide substrates by (methionine sulfoxide)-192-δ-chymotrypsin (MS-δ-Cht) shows a four- to fivefold decrease in k_{cat} and unchanged $K_{m(app)}$ with respect to δ-chymotrypsin. Hydrolysis of α-casein by MS-δ-Cht shows a similar 4.2-fold decrease in k_{cat}. These results imply that the Met-192 acts differently with substrates that bind only in the primary, S_1, binding site (i.e., $AcPheNH_2$) from those that bind to more extended regions of the enzyme active site. In the binding of $AcPheNH_2$ and $AcTrpNH_2$, the results support a mechanism in which the Met-192 acts to slow the rate of sustrate dissociation from the Michaelis complex to free substrate and enzyme. This is in agreement with the x-ray crystallographic structure of dioxane inhibited α-chymotrypsin (Steitz, T., et al. (1969), *J. Mol. Biol. 46,* 337). However, this mechanism is not apparent when peptide and protein substrates bind. The decrease in k_{cat} on Met-192 modification of approximately fivefold in the hydrolysis of polypeptide substrates shows a small, but significant, catalytic contribution of the Met-192 toward the lowering of the energy of activation for *polypeptide* substrate hydrolysis by chymotrypsin. This may support the crystallographic model of Fersht et al. (Fersht, A., et al. (1973), *Biochemistry 12,* 2035) in which it is proposed that the Met-192 participates in the distortion of bound *polypeptide* substrates toward the reaction transition-state configuration and, thus, plays a role in catalysis. However, if this mechanism occurs, the effect is small, only contributing about 1 kcal/mol to the lowering of the reaction activation energy.

X-Ray crystallographic data on Chtgen-A[1] and active forms of Cht have inferred a role for the Met-192 side chain in the events leading to the formation of active Cht from Chtgen and in the catalysis of peptide substrates by active Cht. For example, the comparison of the crystallographic structures of Chtgen-A and δ-Cht shows a repositioning of the Met-192 side chain from a position buried *into* the potential primary substrate binding site (S_1) in Chtgen, to a surface position in active δ-Cht (Freer et al., 1970). Accordingly, it was proposed that the Met-192 blocks accessibility by substrates to the S_1 site in the zymogen form, and its repositioning on activation leads to the ability of substrates to bind at S_1 and be catalytically hydrolyzed by the enzyme (Freer et al., 1970). A role for the Met-192 side chain in catalysis of peptide and protein substrates of Cht was proposed, based on model building from the known x-ray crystallographic structures of α-Cht and its protein inhibitor PTI (Fersht et al., 1973). The model indicated that the binding of the lowest energy conformational state of the peptide substrate to the enzyme active site would result in van der Waals repulsive contacts between the Met-192 side chain carbon atoms and the carbonyl group of the amino acid just C-terminal to the reactive peptide bond in the substrate. These van der Waals interactions, with that between the Ser-195 side chain atoms and the carbonyl group of the reactive bond in the substrate, were proposed to act as a vise to distort the carbonyl carbon of the reactive peptide bond from a ground sp^2 configuration toward the sp^3 configuration of the transition state for the reaction (Fersht et al., 1973). Accordingly, the Met-192 may have a significant catalytic role in the hydrolysis of polypeptide substrates by Cht. The appearance of a Gln side chain of similar length to Met at position 192 in trypsin, procine elastase, and bovine thrombin (Dayhoff et al., 1969) may support a similar role for Gln in these other proteases. In support of this model, it has been reported that MS-α-Cht has a slower autocatalysis rate than native α-Cht (Taylor et al., 1973). Also, the x-ray crystallographic structure of the complex formed between bovine pancreatic trypsin inhibitor and trypsin shows a distortion of the reactive carbonyl carbon in PTI toward a sp^3 tetrahedral configuration, in support of the above model (Ruhlmann et al., 1973).

According to the model of Fersht et al., the vise effect between the Ser-195 and Met-192 cannot occur on the binding of small mono(amino acid) substrates that bind only into the S_1 (primary) binding subsite of the enzyme active site without extending into the S_1'-S_2' subsite of the enzyme. In the x-ray crystallographic structure of dioxane-inhibited α-Cht, in which the molecule of dioxane is bound in the S_1 subsite, the Met-192 side chain folds over the bound inhibitor molecule apparently "holding" the molecule into the primary binding site and thus acting as a sort of door to the S_1 site (Steitz et al., 1969). A similar position for the homologous Gln is observed in the x-ray crystallographic structure of benzamidine-inhibited trypsin (Krieger et al., 1974).

It has previously been shown that oxidation of the Met-192 to methionine sulfoxide reduces the rate of Cht hydrolysis of small mono(amino acid) ester derivatives by a factor of two to three (Koshland et al., 1962; Lawson and Schramm, 1965; Knowles, 1965). However, the effect of Met-192 modification

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[1] Abbreviations: Cbz, benzyloxycarbonyl; Chtgen, chymotrypsinogen; Cht, chymotrypsin; MS-Cht, (methionine sulfoxide)-192-δ-chymotrypsin; PTI, pancreatic trypsin inhibitor; BrNAP, α-bromo-4-nitroacetophenone; DMF, dimethylformamide. S_i refers to the amino acid binding site in the active site for residue P_i of a polypeptide substrate, according to the nomenclature of Schecter and Berger (1967). Based on this nomenclature, a hexapeptide substrate P_3-P_3' would productively align with subsites S_3-S_3' of the enzyme active site, with the substrate's hydrolyzable bond between S_1-S_1'. In eq 1 only, P_1 and P_2 refer to the first and second products of the enzymic reaction, in accordance with standard chymotrypsin nomenclature.

TABLE I: Initial Rates of Hydrolysis of Peptide and Amide Substrates, at pH 7.8[a] and 25 °C.

Substrate	(S) × 10^2 (M)	v_{obsd} × 10^8 (M s^{-1}) (δ-Cht) 1.5 × 10^{-7} M	(MS-δ-Cht) 1.5 × 10^{-7} M
AcPheAlaNH$_2$	0.23	2.41 (1)[b]	
	0.30		0.51 (1)
	0.40		0.70 (1)
	0.68	5.57 (1)	0.97 (1)
	1.35	10.4 (1)	
	1.50		1.71 (1)
	2.50	12.7 (1)	2.52 (1)
		(δ-Cht) 1.5 × 10^{-6} M	(MS-δ-Cht) 1.3 × 10^{-6} M
AcPheGlyNH$_2$	0.30	2.07 (2)	0.44 (4)
	0.40	2.68 (2)	0.69 (4)
	0.60	3.14 (2)	0.82 (2)
	1.00	4.41 (2)	0.96 (2)
	1.40	4.75 (2)	1.15 (2)
	2.80	7.89 (2)	1.63 (2)
AcPheNH$_2$	0.40		0.50 (1)
	0.50	1.41 (1)	0.54 (1)
	0.67	1.90 (1)	0.79 (1)
	1.00	2.43 (1)	1.07 (1)
	2.00		1.79 (1)
	3.00	3.61 (1)	2.43 (1)
	5.00	5.35 (1)	

[a] In 0.1 M $CaCl_2$ and 0.1 M sodium carbonate. [b] Parentheses denote the number of determinations of which the average value was reported.

has not previously been directly evaluated with respect to peptidase rates with polypeptide substrates. This is now of interest because of the role proposed for the Met-192 from the x-ray crystallographic data in the hydrolysis of polypeptide substrates of the enzyme. In this paper we report on changes in peptidase and amidase rates on modification of the Met-192 to methionine sulfoxide.

Experimental Section

Bovine-δ-Cht (CDD OGA) and bovine α-casein were obtained from Worthington Biochemical Corp. α-Bromo-4-nitroacetophenone was obtained from Alfred Bader Chemical Co. and recrystallized from $CHCl_3$–hexane, mp 95–98 °C.

Cbz-Phe-Ala-OEt. To a solution of Cbz-L-Phe (2.5 g, 8.35 mmol) and 0.97 ml (8.65 mmol) of *N*-methylmorpholine in 40 ml of DMF at −15 °C was added 1.15 ml (8.65 mmol) of isobutyl chloroformate, the solution was stirred 30 min, and 1.3 g (8.5 mmol) of HCl-L-Ala-OEt and 0.97 ml (8.6 mmol) of *N*-methylmorpholine in DMF were added. The solution was stirred overnight while slowly warming to room temperature, *N*-methylmorpholine hydrochloride filtered from the solution, the solvent evaporated under vacuum, and the residue dissolved in 100 ml of ethyl acetate, filtered, and washed three times with cold 0.1 N HCl, 4% $NaHCO_3$, and brine. The ethyl acetate solution was dried over $MgSO_4$ (anhydrous), filtered, condensed under vacuum to 60 ml, and stored overnight at °C: yield of product, 2.21 g (61% yield); mp 124.0–125.5 °C; TLC R_f 0.88 (97:3, $CHCl_3$:MeOH), single spot visualized with I_2 vapor, ninhydrin negative.

Cbz-Phe-Gly-OEt was prepared by identical procedures as above starting with 20 g of Cbz-L-Phe and 9.4 g of HCl-Gly-OEt: yield of 18.9 g (67% yield); mp 107.0–108.0 °C (lit. mp 108–110 °C (Gorindachari et al., 1966)); TLC R_f 0.94 (97:3, $CHCl_3$:MeOH) single spot visualized with I_2 vapor, ninhydrin negative.

N-Acetoxysuccinimide. To *N*-hydroxysuccinimide (5 g, 43.5 mmol) in 150 ml of acetone was added 4.1 ml (43.5 mmol) of acetic anhydride dropwise at room temperature with stirring. After 160 min the solvent was evaporated under vacuum, the residue dissolved in 150 ml of absolute ethanol at 45 °C, and the solution cooled to 4 °C, yielding 5.39 g (88% yield) of product, mp 132–133 °C (lit. mp 132–133 °C (Baumann et al., 1973)).

Ac-Phe-Ala-OEt. A solution of Cbz-Phe-Ala-OEt (1.85 g, 4.25 mmol), 0.66 ml of 6.44 N HCl, and 1 g of 5% Pd on carbon in 250 ml of *tert*-butyl alcohol was placed under 20 psi hydrogen in a Parr hydrogenator overnight. The solution was filtered over Celite, the solvent evaporated under vacuum, and the residue desiccated, yielding solid HCl-Phe-Ala-OEt. This residue was dissolved in 43 ml of DMF, 0.48 ml (4.25 mmol) of *N*-methylmorpholine and 0.6 g (4.25 mmol) of *N*-acetoxysuccinimide were added, and the reaction mixture was stirred 4 h at room temperature, evaporated to dryness under vacuum, redissolved in ethyl acetate, washed three times with cold 0.1 N HCl, 4% $NaHCO_3$, and brine, dried over $MgSO_4$ (anhydrous), and filtered, and hexanes were added to give product: yield of 0.65 g (45% yield); mp 153.5–155.0 °C; TLC R_f 0.88 (97:3, $CHCl_3$:MeOH), single spot visualized with I_2 vapor, ninhydrin negative.

Ac-Phe-Gly-OEt was prepared by identical procedures as for Ac-Phe-Ala-Et, starting with 3.2 mmol of Cbz-Phe-Gly-OEt. Yield of first crop was 3.26 g (31% yield): mp 140–142 °C (lit. mp 133–135 °C (Baumann et al., 1973)); TLC R_f 0.52 ($CHCl_3$:MeOH, 97:3), visualized with I_2 vapor, single spot, ninhydrin negative.

Ac-Phe-Ala-NH$_2$. Ammonia gas (anhydrous) was bubbled into a solution of 0.65 g (1.9 mmol) of Ac-Phe-Ala-OEt in methanol for 3.5 h at 0 °C, left 2 days at room temperature, the solvent evaporated under vacuum, and the product crystallized from methanol, yielding 0.46 g (87% yield): mp 246–248 °C (lit. mp 242–244 °C (Fastrez and Fersht, 1973)); TLC R_f 0.85 ($CHCl_3$:MeOH, 4:1), single spot visualized with I_2 vapor, ninhydrin negative.

Ac-Phe-Gly-NH$_2$ was prepared by identical procedures as Ac-Phe-Ala-NH$_2$ above starting with 2.5 g of Ac-Phe-Gly-OEt: yield of 97%; mp 182–184 °C (lit. mp 183–184 °C (Baumann et al., 1973)); TLC R_f 0.70 ($CHCl_3$:MeOH, 4:1), single spot visualized with I_2 vapor, ninhydrin negative.

(Methionine sulfoxide)-192-δ-Cht (MS-δ-Cht) was prepared according to previously published procedures (Weiner et al., 1966). The activity decrease on modification was monitored to a constant level after 22 h reaction by spectrophotometric assay with the substrate *N*-acetyl-L-tyrosine ethyl ester (Schwert and Takenaka, 1955). A sample of the product was treated with α-bromo-4-nitroacetophenone (BrNAP) under conditions that lead to the alkylation by BrNAP of unoxidized Met-192 (Sigman et al., 1969; Schultz et al., 1974). However, no absorption increase at 350 nm was observed on treatment of a solution of oxidized protein with BrNAP, indicating complete oxidation of Met-192 to the sulfoxide form. Active site titrations were carried out on MS-δ-Cht and δ-Cht with *N-trans*-cinnamoylimidazole (Schronbaum et al., 1961) and *p*-nitrophenyl acetate (Bender et al., 1966).

Peptidase rate assays were studied in a unit composed of components from a Technicon amino acid analyzer as de-

TABLE II: Kinetic Parameters Found for the Hydrolysis of Peptide and Amide Substrates by Native and (Methione Sulfoxide)-192-δ-Chymotrypsin.[a]

Substrate	Enzyme Form	k_{cat} (s^{-1})	$K_{m(obsd)}$[b] × 10^3 (M)	Ref
$AcPheAlaNH_2$	δ-Cht	1.5 ± 0.2	19 ± 4	
	MS-δ-Cht	0.32 ± 0.04	25 ± 4	
$AcPheGlyNH_2$	δ-Cht	0.065 ± 0.007	11 ± 2	
	MS-δ-Cht	0.015 ± 0.002	8.4 ± 1.8	
$AcPheNH_2$	δ-Cht	0.043 ± 0.004	17 ± 3	
	MS-δ-Cht	0.044 ± 0.006	43 ± 7	
$AcTrpNH_2$	δ-Cht	0.047	4.8 ± 0.2	*d*
	MS-δ-Cht	0.048	10.6 ± 0.4	*d*
α-Casein	δ-Cht	0.160[c]		
	MS-δ-Cht	0.038[c]		

[a] At pH 7.8–7.9 and 25 °C. [b] $K_{m(obsd)}$ assumed equal to K_s as k_2 rate controlling in amide hydrolysis by Cht (see text). [c] k_{cat} units in absorption units per μg of protein per ml per 20 min, at 35 °C. [d] Knowles, 1965.

scribed previously (Lenard et al., 1965). This apparatus continually measures the appearance of free amine with time by constantly abstracting sample from the reaction solution, combining the sample with ninhydrin reagent, and recording the absorption of the resultant solution. A boiling water bath containing 100 ft of Teflon spaghetti tubing (0.095 in. i.d., 0.107 in. o.d.) was used for development of ninhydrin color. The ninhydrin reagent contained 4 ml of Brij-35 per l. of solution. The system was standardized with NH_4Cl solution (0.25 to 2.0 × 10^{-4} M, ε 6.23 × 10^3 M^{-1} cm^{-1}). Reaction rates were measured over the initial 5% of reaction.

The kinetics of α-casein hydrolysis by δ-Cht and MS-δ-Cht were carried out by previously established procedures (Kunitz, 1947; Green et al., 1952; Wu and Laskowski, 1955). According to these procedures, the reaction was carried out for 20 min at 35 °C, 5% trichloroacetic acid added, the precipitate centrifuged down, and the supernatant peptide concentration read at 280 nm with respect to appropriate controls.

Results and Discussion

The initial amidase and peptidase rates obtained for hydrolysis of Ac-Phe-Ala-NH_2, Ac-Phe-Gly-NH_2, and Ac-Phe-NH_2 by δ-Cht and MS-δ-Cht at pH 7.8 are given in Table I. In all substrates the amide bond contributed by the L-Phe is the one hydrolyzed by the enzyme. Plots of $v/(S)$ vs. v were made for each of the substrates with native δ-Cht and MS-δ-Cht. The intercept and slope of these lines obtained by least-square analysis gave the K_m and k_{cat} values for the respective reactions reported in Table II (Hofstee, 1952). The error in K_m and k_{cat} was computed from the standard error of the estimate (Colton, 1974). Previously published values for the hydrolysis of Ac-Trp-NH_2 by α-Cht and MS-α-Cht are also given in Table II(Knowles, 1965). The ΔA_{280} readings proportional to the peptidase rate on α-casein at different concentrations of δ-Cht and MS-δ-Cht were obtained. These data plotted in Figure 1 show a linearity at δ-Cht < 1.5 × 10^{-7} M and MS-δ-Cht < 4 × 10^{-7} M. A k_{cat} was calculated for each of the enzyme forms with α-casein in the regions of linearity, assuming that in this linear region (S) > K_m and accordingly $v = k_{cat}(E_0)$ (Wu and Laskowski, 1955).

The mechanism of Cht-catalyzed hydrolysis of substrates may be depicted by the simple scheme of eq 1

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \xrightarrow{k_{3'}} P_2 + E \qquad (1)$$
$$+$$
$$P_1$$

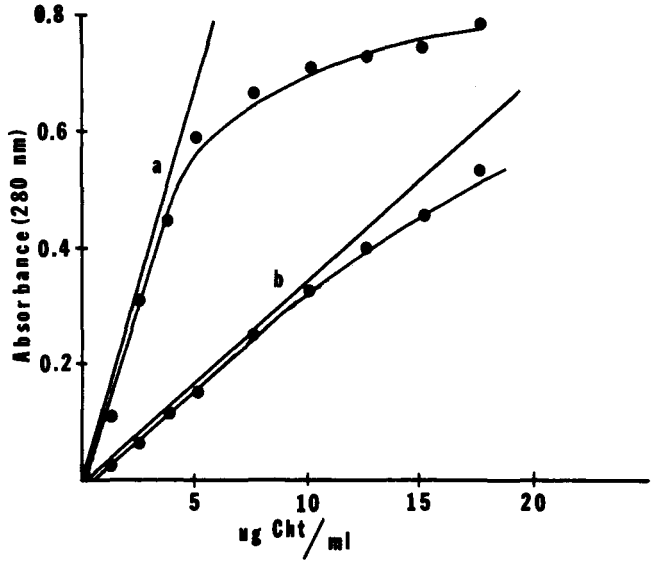

FIGURE 1: Peptide absorbance due to α-casein hydrolysis by (a) δ-Cht and (b) MS-δ-Cht, at 35 °C and pH 7.8.

where ES is the Michaelis complex between enzyme and substrate, ES′ the acylserine intermediate, P_1 the first product of the reaction (ammonia or amine), and P_2 the carboxylic acid product of the hydrolysis reaction (Bender and Kezdy, 1964; Hess, 1971). In amide hydrolysis by Cht, step k_2 is rate controlling and $K_{m(app)} = K_s = k_{-1}/k_1$ (Zerner and Bender, 1964; Hess, 1971). Accordingly, the K_m values found for the enzyme-catalyzed hydrolysis of peptide and amide substrates listed in Table II are true K_s values.

An increase in K_s of twofold was found for the hydrolysis of mono(amino acid) amide substrates ($AcPheNH_2$ and $AcTrpNH_2$) by MS-δ-Cht with respect to δ-Cht, while k_{cat} was apparently unchanged by the Met-192 modification. However, in the hydrolysis of the dipeptide amide substrates k_{cat} was decreased in MS-δ-Cht catalysis by a factor of four- to fivefold, while K_s was apparently unchanged. α-Casein showed a similar decrease in k_{cat} on hydrolysis by MS-δ-Cht as with the dipeptide amide substrates. These data imply that the action of Met-192 is different for mono(amino acid) substrates than for polypeptide substrates. This conclusion is in agreement with inferences that can be made from the x-ray crystallographic data. The crystallographic data for dioxane-inhibited α-Cht show the Met-192 side chain folded over the bound inhibitor in the S_1 pocket, partially blocking its accessibility to the

outside solvent (Steitz et al., 1969).[2] Accordingly, for substrates that bind only into S_1, such as the mono(amino acid) amides, this folding over of the Met-192 side chain may be predicted to affect K_s by decreasing the rate of k_{-1} (eq 1). Accordingly, modification of Met-192 to methionine sulfoxide would be expected to decrease the "holding" effect of the Met side chain leading to an increase in the rate of k_{-1} and a corresponding increase in K_s. Such an effect is seen in Table II in the twofold increase in K_m ($K_m = K_s$) for $AcTrpNH_2$ and $AcPheNH_2$ on modification of δ-Cht to MS-δ-Cht. Further support for the contention that the effect of Met-192 modification is on the rate of k_{-1} rather than k_1 comes from the observation that the change in K_s on Met-192 modification is found only in the binding of the mono(amino acid) amide substrates, and not for all substrates studied. If a change on Met modification occurred in the rate of k_1, the rate change in k_1 would be related to conformational differences between the free forms of δ-Cht and MS-δ-Cht that should be observable in the binding constants for all substrates whether mono(amino acid) derivatives or polypeptide derivatives. It is possible, but not probable, that there is no observable change in the K_s values found for the binding of dipeptide amides to δ-Cht and MS-δ-Cht, due to exact compensation of a change in k_1 by a corresponding change in k_{-1}.

In contrast to the position of the Met-192 indicated from the x-ray crystallographic data for the complex of Cht with a small molecule bound into S_1, model building from the crystallographic structures of PTI and α-Cht implies that, in the Michaelis complex between *polypeptide* substrates and Cht, the Met-192 side chain may assume a position in the S_1' subsite where it can make strong van der Waals contacts with the P_1' residue of the substrates (Fersht et al., 1973). It is argued that this interaction may help to distort the reactive peptide bond in the bound substrate toward the transition state of the reaction, and thus act to lower the energy of activation for the enzymic reaction with respect to the nonenzymic reaction (Fersht et al., 1973). The decreases found in k_{cat} for the MS-δ-Cht hydrolysis of the dipeptide amides and α-casein are in agreement with this hypothesis. However, the decrease of approximately four- to fivefold on modification of Met-192 is small. Comparison of Cht-catalyzed rates of amide hydrolysis with the rates of amide hydrolysis in nonenzyme systems indicates that Cht lowers the energy of activation for peptide bond hydrolysis in specific substrates by approximately 12 kcal/mol (Bender et al., 1964). A factor of approximately fivefold due to the Met-192 represents an effect of 1 kcal/mol toward the total catalytic effect or approximately 8% of the energy of activation difference between the enzymic and nonenzymic hydrolysis. As the effect on k_{cat} is small and the hypothesis based on model building from x-ray crystallographic structures may be considered speculative, it is possible that other small effects such as a change in enzyme–substrate complex solvation or a minor change in active site conformation on Met-192 modification can cause the decrease in k_{cat} of approximately 1 kcal/mol. However, the clear observation that, on modification of Met-192, k_{cat} is not decreased for the mono(amino acid) amide substrates, while it is decreased for peptide and protein substrates, supports the explanation of the crystallographic hypothesis.

[2] A similar position of the Gln-192 is observed in the crystalline structure of benzamidine-inhibited trypsin (Krieger et al., 1974).

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