CATALYSIS BY CHYMOTRYPSINOGEN: INCREASED REACTIVITY DUE TO OXIDATION OF METHIONINE 192

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1. Introduction

Zymogens of certain proteolytic enzymes exhibit catalytic activities which are 4 to 6 orders of magnitude lower than those of the corresponding enzymes [1–9]. We have recently demonstrated [10] that chymotrypsinogen catalyzes the hydrolysis of *p*-nitrophenyl, *p'*-guanidinobenzoate (NPGB) and that the reaction proceeds via an acyl–zymogen intermediate which deacylates only 70 times slower than the corresponding acyl-enzyme. This study, together with kinetic measurements of the rates of reaction of trypsinogen with NPGB [10] and with methane sulfonyl fluoride [11] led us to conclude that the principal reason for the low activity of these zymogens is ineffective substrate binding. Since the orientation of Met 192 of the 'tosyl pocket' of chymotrypsin [12] plays an important role in the binding of substrates, we have explored the effects of chemical modification of this residue on the catalytic activity of chymotrypsinogen. A method for specifically oxidizing Met 192 of chymotrypsinogen to the sulfoxide was recently described by Wasi and Hofmann [13]. We find that the rate of reaction of the modified chymotrypsinogen with *p*-nitrophenyl esters is 7–8 times higher than that of native chymotrypsinogen whereas the pH dependence of deacylation is unchanged. Oxidation of Met 192 also enhances approximately twofold the rate of reaction with DFP. The increased reactivity of the oxidized chymotrypsinogen is primarily due to an improved affinity for these site-specific reagents.

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2. Materials and methods

2.1. *Materials*

p-Nitrophenyl-*p'*-guanidinobenzoate hydrochloride (NPGB) was purchased from Cyclo Chemical Co. *p*-Nitrophenyl-*p'*-(-dimethyl-sulfonio-acetamido) benzoate bromide (NPSA), a specific active site titrant of chymotrypsin [14] was a gift from Dr. E. Shaw. Seven times crystallized bovine chymotrypsinogen A containing only 0.02% of active enzyme was a gift from Dr. K. Kurachi. All other materials were described elsewhere [10].

2.2. *Preparation of Met-0 192-chymotrypsinogen*

The procedure of Wasi and Hofmann [13] was employed with minor modification to selectively oxidize Met 192 to the sulfoxide. Chymotrypsinogen (100 mg) was dissolved in 10 ml of 1 mM HCl containing 2 mM EDTA, adjusted to pH 3.0 with 1 M HCl and diluted with water to a final volume of 19 ml. After the addition of 1 ml H_2O_2 (Baker, 30% v/v) the reaction was allowed to proceed at 22°C for 18 hr. The protein was then desalted on a column (2.5 × 36 cm) of Sephadex G25 in 1 mM HCl and lyophilized. The stoichiometry of the reaction was determined by the technique of 'secondary alkylation' [15] using [^{14}C] iodoacetic acid in 8 M urea (pH 3). Since the incorporation of carboxymethyl groups was 44% of that into chymotrypsinogen controls, it was concluded that of the 2 methionyl residues 1.1 were protected from alkylation and in addition to Met 192 a small fraction of Met 188 was also oxidized. The isolated product is thus a mixture of 85–90% monosulfoxide-chymotrypsinogen and 10–15% disulfoxide-chymotrypsinogen.

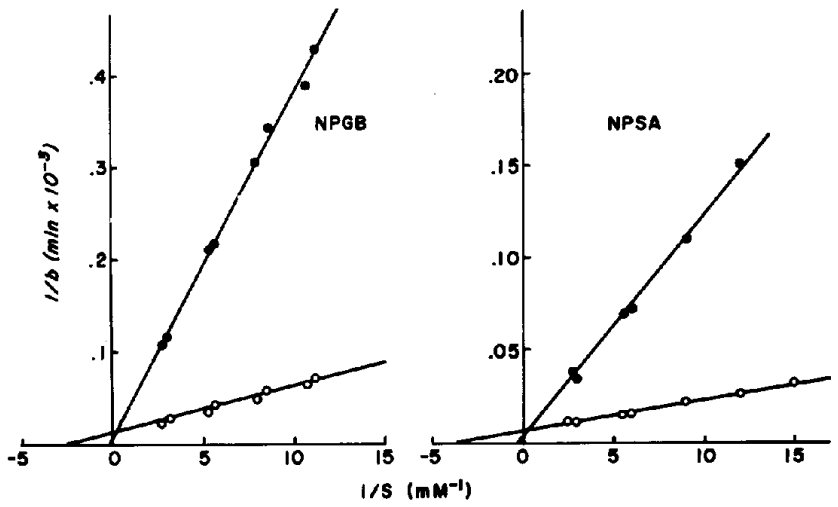

Fig. 1. Reciprocal plot of the reaction of chymotrypsinogen (o) and Met-0 192-chymotrypsinogen (•) with NPSA and NPGB.

2.3. *Reaction of zymogens with NPGB and NPSA*

The reaction mixtures (2.5 ml) were prepared by adding to 0.5 ml chymotrypsinogen or Met-0 192-chymotrypsinogen (0.1 mM) in 1 mM HCl, 1.95 ml 0.1 M PIPES buffer (pH 7.2) containing 0.04 M $CaCl_2$ and 50 μl of 5–20 mM NPGB, or 100 μl of 2–10 mM NPSA, dissolved in dimethylformamide. The reactions were followed in a Cary 14 spectrophotometer at 25°C which recorded at 400 nm the release of *p*-nitrophenol. In control reactions chymotrypsinogen was replaced by DIP-chymotrypsinogen. The extent of acylation was calculated at various times from the difference in absorbance between the reaction and the control. The change in absorbance resulting from the release of 1 mole of *p*-nitrophenol/mole of chymotrypsinogen was determined from the concentration of chymotrypsinogen and from the specific molar absorbance of *p*-nitrophenol in 0.1 N NaOH (ϵ_{400} = 18 000) [16]. The exact concentration of NPGB or NPSA in each experiment was calculated from the absorbance at 400 nm after complete hydrolysis in 0.1 N NaOH.

The pseudo-first order rate constants for acylation were calculated from the initial portion of the semilogarithmic plots assuming that in the initial 'burst' 1 mole of *p*-nitrophenol/mole of zymogen is released. In this initial reaction less than 20% of chymotrypsinogen was acylated and the release of free deacylated zymogen had a negligible effect on the reaction kinetics.

2.4. *Other procedures*

All other procedures, including reaction with DFP, preparation of *p*-guanidinobenzoyl-chymotrypsinogen (*p*GB-chymotrypsinogen), estimation of the extent of acylation and deacylation of the acyl-zymogen at different pH values, were described elsewhere [10].

3. Results

3.1. *Acylation of Met-0 192-chymotrypsinogen and chymotrypsinogen by NPGB and NPSA*

Both NPGB and NPSA are hydrolyzed by chymotrypsinogen and by Met-0 192-chymotrypsinogen. The reactions exhibit a pre-steady state release of nitrophenol followed by steady state hydrolysis.

In order to estimate the apparent dissociation constant (K_s) and the maximal acylation rate (k_2), pseudo-first-order rate constants were determined at various concentrations of NPGB or NPSA. These constants are defined by eq. 1:

$$\frac{1}{b} = \frac{1}{k_2} + \frac{K_s}{k_2[S]_0} \qquad (1)$$

Table 1
Comparison of kinetic properties of chymotrypsinogen and Met-0 192-chymotrypsinogen.

Reaction parameter	Chymotrypsinogen	Met-0 192-chymotrypsinogen
k_{II}[a] of acylation by NPGB ($min^{-1}\cdot M^{-1}$)	24.8	198.0
k_{II}[a] of acylation by NPSA ($min^{-1}\cdot M^{-1}$)	8.2	55.6
k_{II}[a] of phosphorylation by DFP ($min^{-1}\cdot M^{-1}$)	0.21[b]	0.48
k_{3max} of deacylation of *p*GB-zymogen (min^{-1})	0.0089[c]	0.0098
pK_a of the group responsible for pH dependence of deacylation	7.07[c]	7.08

[a] Second order rate constant.
[b] For reaction at pH 8.0 [10].
[c] As previously reported [10].

where b is the pseudo-first-order rate constant for the pre-steady state reaction at a given substrate concentration $[S]_0$ [17]. This equation predicts a linear relationship between $1/b$ and $1/[S]_0$ with intercepts of $-1/K_s$ and $1/k_2$.

A plot of equation (1) using different substrate concentrations (fig. 1) indicates that oxidation of Met-192 increases the reactivity in comparison to the unmodified zymogen. The kinetic parameters for the reaction of Met-0 192-chymotrypsinogen with NPGB and NPSA were calculated using a least squares program and yielded values for K_s of 0.45 mM and 0.29 mM, and for k_2 of 0.09 min^{-1} and 0.016 min^{-1}, respectively. In the case of chymotrypsinogen, the reactions were so slow that only second order rate constants (k_2/K_s) could be calculated from the slope (table 1).

3.2. *Deacylation of pGB-Met-0 192-chymotrypsinogen*

*p*GB-Met-0 192-chymotrypsinogen was prepared and isolated by the method described previously for *p*GB-chymotrypsinogen [10], but the reaction was stopped after 80 min instead of 4 hr. The extent of acylation, as determined by activation of the zymogen at pH 3.5 [10] was 76%.

The rates of deacylation of *p*GB-Met-0 192-chymotrypsinogen were measured in the pH range 6.3–7.6 at 5 different pH values. The apparent first-order rate constants, k_3, at each pH were multiplied by the respective (H^+) and plotted against k_3 according to the linear equation:

$$k_3(H^+) = K_a k_{max} - K_a k_3 \quad (2)$$

A linear relation was obtained using a least squares program, thus indicating that deacylation was dependent on the ionization of a single group. From the slope and the ordinate intercept, values of $pK_a = 7.08$ and $k_{max} = 0.0098$ min^{-1}, respectively, were obtained.

3.3. *Inactivation of Met-0 192-chymotrypsinogen by DFP*

The reaction was carried out at pH 8.0 as described previously [10] using two different initial DFP concentrations (23 mM and 4.6 mM). Second-order rate constants (k_{II}) were calculated from semi-logarithmic plots using an integrated equation that corrects for spontaneous hydrolysis and progressive dilution [8]. The calculated values of k_{II} were 0.474 $min^{-1}\cdot M^{-1}$ and 0.480 $min^{-1}\cdot M^{-1}$. The similarity of these values of k_{II} indicates that these two concentrations of DFP were insufficient to saturate the binding site of the zymogen.

4. Discussion

Table 1 summarizes the kinetic properties of native and oxidized chymotrypsinogen. Conversion of Met-192 to the sulfoxide increased the second-order rate constant (k_{II}) of acylation of chymotrypsinogen by NPGB or NPSA 7- to 8-fold, whereas the rate of reaction with DFP increased only 2-fold. Although direct comparison of the kinetic parameters, k_{cat} and K_s, of native and oxidized chymotrypsinogen was not possible, it is evident from fig. 1 that the increase in the second order rate constant upon oxidation of Met-192 is accompanied by a decrease of K_s, probably due to an increased affinity for the substrate. This explanation is also supported by the observations that the rate of deacylation of the acyl zymogen, and the pK_a for the ionization of the single group responsible for the pH dependence of this reaction, are essentially the same in the native and the oxidized zymogens.

Met 192 is one of the few amino acid residues

which are translocated during the process of activation of chymotrypsinogen. In this process the main chain rotates 180° about the bond between the carbonyl- and the alpha-carbon of Asp 194, and promotes the formation of a new ion pair between the alpha amino group of Ile 16 and the beta-carboxyl group of Asp 194. The same rotation shifts Met 192 from a buried position to the surface of the molecule, thus contributing to the shape of a 'specificity cavity' which is imperfectly developed in the zymogen [18]. While the rate of oxidation of Met 192 in alpha-chymotrypsin is the same as that of free methionine, the oxidation rate of this residue in chymotrypsinogen is 40 times slower at 4–25°C [13]. Wasi and Hofmann concluded that the low reactivity of Met 192 in the zymogen was due to 'hindered accessibility' of this residue to the reagent rather than the consequence of an equilibrium between a folded and unfolded form of the zymogen.

In terms of the present results, it may be suggested that oxidation increases the size of the side chain of Met 192 and that the resulting shift in its position induces a conformational change in the direction of the zymogen–enzyme conversion. Alternatively, oxidation of Met 192 may enhance the affinity of site-specific reagents by inducing a conformational transition at a site other than the 'tosyl pocket.' The present data do not permit a choice between these or other explanations. Since Met-O 192-chymotrypsinogen is a stable derivative, crystallographic analysis could elucidate the structural changes due to oxidation and the specific mode of binding of pseudo substrates to the native and oxidized zymogens. The half-life of *p*-guanidinobenzoyl derivatives of the zymogens at pH 4 is estimated to be approx. 1000 hr (table 1) which is sufficiently long for crystallographic analysis.

Acknowledgements

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