Microscopic rate-constants for substrate binding and acylation in cold-adaptation of trypsin I from Atlantic cod

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Abstract Temperature imposes limits on where life can thrive and this is evident in the evolution of the basic structural properties of proteins. Cold-adaptation of enzymes is one example, where the catalytic rate constant (k\textsubscript{cat}) is increased compared with hot-acclimated homologous under identical assay conditions. Trypsin I from Atlantic cod (Gadus morhua) has catalytic efficiency (k\textsubscript{cat}/K\textsubscript{m}) for amide hydrolysis that is 17-fold larger than observed for bovine trypsin. Here, the individual rate-constants for association of substrate (k\textsubscript{1}), dissociation of substrate (k\textsubscript{-1}), and acylation of the enzyme (k\textsubscript{2}) have been determined using benzoyl-Arg-p-nitroanilide or benzoyloxycarbonyl-Gly-Pro-Arg-p-nitroanilide as substrates. Rather unexpectedly, by far the largest difference (37-fold increase) was observed in k\textsubscript{1}, the rate constant for binding of substrate. The cold-adaptation of the dissociation and catalytic steps were not as prominent (increased by 3.7-fold). The length of substrate did have an effect by increasing the reaction rate by 70-fold, and again, the step most affected was the initial binding-step.

Keywords: Cold-adaptation; Psychrophilic; Serine proteinase; Kinetics; Rate-constants; Gadus morhua

1. Introduction

The adaptation of living organisms to extremes of temperature is of great biological and practical interest. Cold-adapted enzymes have evolved to function well at low temperatures through modifications of structural properties in comparison with homologous enzymes found in warm-blooded species [1–4]. It is believed that more rapid binding and release of reactants, coupled with easier flow through the catalytic steps, occurs by more facile (local) movements in the structure. How this is achieved by structural adjustments is not yet fully understood, but one common characteristic observed in these more active variants is a lower temperature stability that supports the notion of a lesser molecular adhesion in the structure.

Cold-adaptation is defined in terms of steady-state rate measurements by comparing catalytic efficiencies (k\textsubscript{cat}/K\textsubscript{m}) of homologous enzymes. A more detailed information about microscopic rate constants would add to our understanding of cold-adaptation. A method was recently described for determine individual rate-constants for substrate hydrolysis by serine proteinases, where the rate-constants for substrate binding, substrate dissociation, and acylation are derived from the temperature dependence of the Michaelis–Menten parameters. The method also yields the activation energies for these molecular events [5,6]. Serine proteinases are of widespread occurrence with diverse biological and physiological functions, including digestive and degradative processes, blood coagulation, fibrinolysis, complement activation, and embryonic development [7–10]. Furthermore, structural movement is an integral part of their mechanism [11–14]. Digestive serine proteinases from cold-adapted animals have been much studied in an effort to understand the basis of their improved catalytic mechanism [15–21], but more factual information is needed. The catalytic mechanism of the serine proteinases is characterized by the presence of one uniquely reactive serine side-chain and it goes through a covalent intermediate, separated by an acylation step that cleaves the peptide bond, followed by a hydrolytic deacylation step of the covalently bound intermediate [7]. With amide substrates the rate-determining step is acylation, whereas with ester substrates the deacylation step is slower [22].

Trypsin belongs to the largest family of serine peptidases and is one of the best studied example. We have previously isolated three trypsin isoenzymes in pure form from Atlantic cod and reported the Michaelis–Menten constants for hydrolysis of the ester substrate Tosyl-Arg methyl ester and the amide substrate Bz-Arg-pNA [17]. The catalytic efficient (k\textsubscript{cat}/K\textsubscript{m}) of cod trypsin I was about 17-fold higher than observed with bovine trypsin with the more physiologically relevant amide (Bz-Arg-pNA) as substrate. The turnover number (k\textsubscript{cat}) for cod trypsin I was about double that of bovine trypsin and the K\textsubscript{m} value one-eighth. Since cold-adaptation of other trypsins appeared to be also directed both to substrate affinity as well as improved catalytic ability [16,19,20,23], we were interested in finding out to which microscopic rate constants this difference could be traced, and thus apply the method mentioned above to a cold-adapted enzyme for the first time. A tripeptide substrate was used in addition to Bz-Arg-pNA to assess the role of an extended substrate binding site.

2. Materials and methods

2.1. Materials

Atlantic cod trypsin I was purified as previously described [17]. Bovine trypsin Type-III, N-benzoyl-L-arginine-p-nitroanilide (Bz-Arg-pNA), benzoyloxycarbonyl-glycine-proline-arginine-p-nitroanilide (Z-Gly-Pro-Arg-pNA) and other chemicals for preparation of buffers were obtained from Sigma Chem. Co. (USA).

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2.2. Activity measurements

The substrates were dissolved at 12.5 mM in dimethylsulfoxide (Me2SO) and used at a final concentration of 0.025–0.5 mM (Bz-Arg-pNA) or 0.0125–0.125 mM (Z-Gly-Pro-Arg-pNA). The assay medium contained 50 mM Tris/HCl (pH 8.0) and 10 mM CaCl2. The final concentration of Me2SO was 2% (v/v). For assays at various temperatures, the enzyme sample (0.01 ml) was added to a pre-heated cuvette (0.99 ml) and the rate of hydrolysis for the next 5 s (Z-Gly-Pro-Arg-pNA) or 30 s (Bz-Arg-pNA) determined after a 5 s equilibrium period. Corrections were made for the effect of temperature on pH. An extinction coefficient for p-nitroanilide of 8800 M−1 cm−1 at 410 nm was used [24].

2.3. Data handling

The steady-state kinetic constants $k_{cat}$ and $K_m$ were determined using non-linear fits to the Michaelis–Menten equation. The experiments were repeated several times and no individual result exceeded more than 10% off the average value.

A minimal reaction scheme for a serine proteinase may be described by four rate-constants:

$$E + S \xrightarrow{k_1} ES \xrightarrow{k_{-1}} E- P_1 + P_1 \xrightarrow{k_2} E + P_2. \quad (1)$$

The acylation step ($k_2$) is typically rate-determining for amide substrates [22], and hydrolysis of the E–P2 intermediate ($k_{-1}$) is effectively irreversible in water. Obtaining these four rate-constants will, therefore, suffice to describe the mechanism. The Michaelis–Menten parameters that are accessible to direct experimental measurement are composite functions of the individual kinetic rates of scheme (1). $K_{cat}$ is not dependent on substrate binding or product dissociation ($k_{cat} = k_2/k_1 + k_{-1}$), whereas the selectivity constant, $s = k_2/k_1$ ($k_{-1} = k_1$), measures catalytic efficiency ($k_{cat}/K_m$), does not depend on the deacylation rate. Because four independent rate constants define two independent Michaelis–Menten parameters ($k_{cat}$ and $K_m$), knowledge of $s$ and $k_{cat}$ from experimental measurements is not sufficient to completely resolve the various steps of the kinetic mechanism in scheme (1). The temperature dependence of rate constants obeys the Arrhenius’s plot ($\ln(k_{cat})$ vs. $1/T$) and Fig. 2 shows the comparative plot of $\ln(s)$ vs. $1/T$ for cod trypsin I and bovine trypsin. The Arrhenius plots were linear that confirms the acylation step as rate-limiting for both substrates (Fig. 1). All the $\ln(s)$ vs. $1/T$ plots had a distinct maximum (Fig. 2). The slope changed from negative at low temperatures, where information is gathered on $k_1$ (and $E_1$), to a positive slope at high temperature, where information is obtained on $k_1 + k_2/k_{-1}$ (and $E_{-1}$, $E_1$ and $E_2$) [5].

Table 1 summarizes the individual rate-constants and activation energies. The steady-state values obtained for $k_{cat}$ and $K_m$ at 25 °C compare well with literature values regarding Bz-Arg-pNA [17], but values for Z-Gly-Pro-Arg-pNA have apparently not been published before for cold-water fish trypsins. The substrate Bz-Arg-pNA commonly used for studying trypsin can be considered as a rather poor substrate compared with its natural substrates, due to its small contact area with the enzyme. Only one amino acid is bound in the $P_1$-site in addition to the benzoyl group in the $P_2$-site, whereas the leaving group is a small $p$-nitroanilide group initially bound in the $P_1$-site. The data obtained here show that the difference in $k_2$ goes a long way to explain the previously observed $k_{cat}$ values [17] of 4.0 s$^{-1}$ for cod trypsin I (here 3.44 s$^{-1}$) and 2.0 s$^{-1}$ for bovine trypsin (here 1.13 s$^{-1}$). The dissociation rate-constant, $k_{-1}$, was larger for cod trypsin I at 0.41 s$^{-1}$ compared with 0.11 s$^{-1}$. This is in keeping with the idea that cold-active enzymes bind substrates with lower affinities due to more structural flexibility and dynamic movement in the active-site area. The ratio for the forward going reaction toward the acyl-enzyme intermediate, $k_2/k_{-1}$, is about 0.9 for both enzymes. The substrate equilibrium dissociation constants ($K_s$) are 4.0 × 10$^{-5}$ M$^{-1}$ s$^{-1}$ and 3.3 × 10$^{-6}$ M$^{-1}$ s$^{-1}$ for the bovine trypsin and cod trypsin I, respectively. Thus, a previously observed 17-fold difference in catalytic efficiency [17] can now be largely traced to the much faster binding step of substrate into the cod trypsin active site: 1.2 × 10$^5$ M$^{-1}$ s$^{-1}$ vs. 3.2 × 10$^3$ M$^{-1}$ s$^{-1}$. Activation energies ($E_a$) can be converted to enthalpy values using the relation $\Delta H^\circ = E_a - RT$. Furthermore, the Gibbs
Pro-Arg-conditions were 50 mM Tris/HCl (pH 8.0) and 10 mM CaCl₂ with final $p$ during a 30 s assay period with Bz-Arg-

D/C₁₇₆ is larger at $10^{-50}$ and $10^{-50}$ for Bz-Arg only with Bz-Arg-pNA (open squares) and Z-Gly-Pro-Arg-
pNA (black squares) and Z-Gly-Pro-Arg-pNA (black circles), whereas bovine trypsin only with Bz-Arg-pNA (open squares). Z-Gly-Pro-Arg-pNA assays were conducted for 5 s periods. Experimental conditions were 50 mM Tris/HCl (pH 8.0) and 10 mM CaCl₂ with final concentration of Me₂SO at 2% (v/v). The temperature was in the range 10–50 °C.

free energy values can be calculated using the relationship $\Delta G ^ {\circ} = RT \ln \left(23.76 + \ln k_{\text{cat}}^\circ \right)$ kJ/mol, and the entropy value as $\Delta S ^ {\circ} = (\Delta H ^ {\circ} - \Delta G ^ {\circ})/T$ assuming equilibrium between ground-state (ES complex) and the transition-state (ES# complex) of the substrate [25]. The comparison of $\Delta G ^ {\circ}$ values shows for Bz-Arg-pNA that the difference is small, 11.06 and 13.82 kJ/mol for cod and bovine trypsin, respectively. The difference $\Delta (\Delta G ^ {\circ})_{\text{cod-bovine}}$ is negative as expected for cold-adaptation. Furthermore, the enthalpy difference, $\Delta (\Delta H ^ {\circ})_{\text{cod-bovine}}$ is larger at $-7.27$ kJ/mol than the entropy difference, $\Delta (\Delta S ^ {\circ})_{\text{cod-bovine}}$, at $-4.51$ kJ/mol at $25$ °C. Most notably, both values are negative indicating an enthalpy–entropy compensation. Thus, catalysis requires a greater overall reduction in structural entropy as a more flexible enzyme takes hold of the substrate for catalysis. This is commonly observed for cold-adapted enzymes and is likely a general strategy to fulfill the free-energy requirement and at the same time lowering the $\Delta H$ needed for breaking weak interactions in the course of the catalytic event by having them fewer [25].

We were interested in seeing how the length of the substrate towards the N-terminus might alter the relative magnitude of the microscopic rate-constants in cod trypsin catalysis. Table 1 shows results obtained with Z-Gly-Pro-Arg-pNA, a substrate known to give a much faster $k_{\text{cat}}$ than Bz-Arg-pNA with mammalian trypsins. The difference with cod trypsin I was about 50-fold higher $k_{\text{cat}}$ and a $K_{m}$ that was 50% reduced. Both the association rate ($k_{1}$) and dissociation rate ($k_{-1}$) were increased. The presence of Pro in the $P_2$ position, but not elsewhere, aligns the substrates for productive binding, thus helping catalysis [26]. This was also apparent with cod trypsin I as $k_{2}$ was increased 70-fold compared with Bz-Arg-pNA (Table 1). The effect of the leaving group has been studied with rat trypsin using Z-Gly-Pro-Arg as substrate. For wild-type rat trypsin, the $k_{\text{cat}}$ remained approximately $50-66$ s⁻¹ regardless of whether the leaving group was benzyl, thioester, $p$-nitroanilide, or aminomethylcoumarin [27]. This indicated that acylation is not rate-limiting, and overall rate is determined by hydrolysis of the acyl-enzyme covalent intermediate. With the fluorogenic variant Z-Gly-Pro-Arg-7-amino-4-methylcoumarin, rat trypsin displayed a $k_{\text{cat}}$ of 55.7 ± 5 s⁻¹ and a $K_{m}$ of 14.1 ± 1.3 μM [28]. The Michaelis–Menten constant and turnover number of the human cationic trypsin with Z-Gly-Pro-Arg-pNA were reported as $K_{m}$ 27 ± 3 μM and $k_{\text{cat}}$ 99 ± 5 at 22 °C [29], and for anionic human trypsin as $K_{m}$ 15 ± 1 μM and $k_{\text{cat}}$ 50 ± 1 s⁻¹ at 22 °C [30]. These values serve well for comparison with the values for cod trypsin obtained in this study; $K_{m}$ 17 ± 3 μM and $k_{\text{cat}}$ 172 ± 35 s⁻¹ at 25 °C. With the longer substrate, the cold-adapted cod trypsin is mainly different with respect to the apparent $k_{\text{cat}}$ value rather than $K_{m}$. This indicates that adaptation is directed at the hydrolysis step rather than
Binding of protein substrates to serine proteinases is dominated by the primary specificity pocket designated S1 by convention [31]. The P1 residue also determines the operation of the catalytic triad of serine proteinases during hydrolysis of acyl-envelope intermediates [32,33]. In trypsins, a lysine or an arginine in substrate position P1 binds to the hydrophobic walls of the S1-pocket, an interaction greatly reinforced by the Asp189 in its bottom. Numerous studies have shown, however, that the specificity of serine proteinases is much more complex than this description implies [34,35]. In relation to cold-adaptation, anionic salmon trypsin displayed 100-fold higher association constants ($K_a$) than bovine trypsin for 17 P1-variants of bovine pancreatic trypsin inhibitor. A P1 Gly variant bound to both cod and bovine trypsin with $K_a$ of the order of 6 kcal/mol suggesting a considerable and similar energy contribution from secondary binding-sites [36]. The effects of substitutions at the P1 position on the association constants were found to be very large, comprising seven orders of magnitude for trypsin [37]. Numerous studies iterate the fact that higher catalytic efficiency is the hallmark of cold-adaptation, and is most frequently achieved by an increase in $k_{cat}$ rather than affecting $K_m$ [4,38,39]. However, the most abundant cold-water fish trypsin displayed up to 35-fold higher catalytic efficiency ($k_{cat}/K_m$) than bovine trypsin [19] largely based on lower $K_m$. In fact, high affinity for substrate has been generally observed for cold-adapted trypsins in comparison with mammalian homologues [20] even using a single-amino acid synthetic substrate such as Bz-Arg-pNA. This suggests that stronger substrate affinities arise mainly from stronger interactions with the S1 site. It has been proposed that the altered electrostatic potential of the S1 site leading to enhanced substrate binding is caused by non-conserved acidic residues outside the S1 site giving the active-site more negative charge in cold-adapted trypsins [40]. In that work, the kinetic constants were determined using Tosyl-Gly-Pro-Arg-pNA at pH 8.3 with $k_{cat}$ 30.4 s$^{-1}$ and 88.0 s$^{-1}$ and $K_m$ 0.9 and 5.19 μM for anionic salmon trypsin and bovine trypsin, respectively. The lower $k_{cat}$ value for the salmon trypsin is somewhat unexpected, but $k_{cat}/K_m$ was about double for the cod enzyme; 3.39 × 10$^{-7}$ s$^{-1}$ M$^{-1}$ compared with 1.70 × 10$^{-7}$ s$^{-1}$ M$^{-1}$ [40].

Cold-active trypsins have in common an anionic surface potential (acidic pH) [17,20,41]. This has led to the proposal that the surface electrostatic potential assists in promoting substrate binding in trypsin, and thus catalysis [42–45], since molecular dynamics calculation did not reveal any great differences in the potential for dynamic movement in trypsins overall [46,47]. However, it was concluded that an apparent higher deformability of the active site of anionic salmon trypsin might lower the activation energy for ligand binding and for catalysis, increasing binding affinity and catalytic efficiency compared with cationic bovine trypsin. Similar to the mammalian trypsins that are also cationic, cationic fish trypsins (pI > 9) did not show the anionic trypsin features of high catalytic efficiency [19] and some anionic trypsins may depend more on increasing $k_{cat}$ for adaptation [15].

The natural trypsin substrates form an antiparallel beta-sheet with the seven amino acid binding-site of trypsin, and individual active-site residues do consequently have strong effects on kinetic properties, in particular the P1 site. Replacement of the aspartic residue (Asp-189) at the base of the substrate binding-pocket of rat trypsin by serine decreased the activity of Ser189-trypsin on lysyl and arginyl substrates (within succinyl-Ala-Ala-Pro-Xaa-7-amino-4-methyl-coumarin) by about 5 orders of magnitude whilst its $K_m$ values increased only 2–6-fold. In contrast, Ser189-trypsin was 10–50 times more active on the less preferred, chromotryptsin-type substrates (tyrosyl, phenylalanly, leucyl, and tryptophanyl). The relative binding-energies in the transition state indicated that these side-chain interactions become prominent during the transition of the Michaelis-complex to the tetrahedral transition-state complex [48]. This may be part of the “binding” step prior to the nucleophilic attack form the active serine that is accelerated in cod trypsin I. Comparison with the other two trypsin variants found in cod would suggest that the differences in the active site amino acid sequence promotes its special properties, but further comparisons must await the sequencing of those variants. Key variations in residues may also be found elsewhere in the structure (for example in loop regions) promoting subtle positioning and mobility of the catalytically competent active site configuration. Enhanced primary binding has been suggested as an important factor in enhancing the catalytic efficiency of salmon trypsin relative to bovine trypsin [19] and our results.

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**Table 1**

Kinetic rate constants at 25 °C and activation energies for Bz-Arg-pNA and for Z-Gly-Pro-Arg-pNA hydrolysis by bovine and cod trypsin I

<table>
<thead>
<tr>
<th></th>
<th>Cod trypsin I</th>
<th>Z-Gly-Pro-Arg-pNA</th>
<th>Bovine trypsin</th>
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</thead>
<tbody>
<tr>
<td><strong>Rate constants</strong></td>
<td></td>
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<tr>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>3.44 ± 0.20</td>
<td>172.8 ± 35.4</td>
<td>1.13 ± 0.02</td>
</tr>
<tr>
<td>$K_m$ (mM)</td>
<td>0.034 ± 0.002</td>
<td>0.017 ± 0.003</td>
<td>0.430 ± 0.012</td>
</tr>
<tr>
<td>$k_1$ (M$^{-1}$ s$^{-1}$)</td>
<td>1.22 ± (0.12)×10$^5$</td>
<td>2.25 ± (0.19)×10$^7$</td>
<td>3.23 ± (0.39)×10$^3$</td>
</tr>
<tr>
<td>$k_3$ (s$^{-1}$)</td>
<td>0.41 ± 0.17</td>
<td>134.7 ± 30.3</td>
<td>0.11 ± 0.04</td>
</tr>
<tr>
<td>$k_2$ (s$^{-1}$)</td>
<td>3.37 ± 0.07</td>
<td>238.0 ± 4.7</td>
<td>0.93 ± 0.02</td>
</tr>
<tr>
<td>$k_3$: $k_1/k_2$</td>
<td>0.12 ± 0.05</td>
<td>0.57 ± 0.11</td>
<td>430.6 ± 0.3</td>
</tr>
</tbody>
</table>

| **Activation energies** |               |                   |               |
| $E_1$ (kJ/mol)          | 9.07 ± 0.5    | 1.27 ± 0.4        | 33.07 ± 0.4   |
| $E_2$ (kJ/mol)          | 80.60 ± 4.0   | 94.54 ± 1.4       | 110.07 ± 6.6  |
| $\Delta G^\circ$ (kJ/mol) | 32.61 ± 0.9   | 63.00 ± 1.2       | 39.88 ± 3.7   |
| $\Delta H^\circ$ (kJ/mol) | 11.06         | 1.35              | 13.82         |
| $\Delta S^\circ$ (J mol$^{-1}$ K$^{-1}$) | 30.13         | 60.52             | 37.40         |
| $\Delta S^\circ$ (J mol$^{-1}$ K$^{-1}$) | 0.0640        | 0.20              | 0.0791        |

Non-linear regression analysis was used to obtain individual rate constants.
provide direct measurements that confirm that for a small substrate but not for the larger substrate.

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References


