pH-dependent hysteretic behaviour of human myeloblastin (leucocyte proteinase 3)

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Human myeloblastin (leucocyte proteinase 3) showed a very slow approach to the steady-state velocity when the pH was rapidly increased from 3.2 to 7.0. The kinetic mechanism of this hysteretic process was interpreted as a slow conformational change of myeloblastin from an inactive form at acidic pH to the active form at neutral pH. The transition between the two enzyme forms could occur spontaneously in the absence of substrates with a first-order rate constant of 0.0033 s^{-1} . In the presence of peptide substrates activation occurred more rapidly:

the observed rate constant was linearly dependent upon the substrate concentration and contained a contribution of the spontaneous as well as of the substrate-dependent process, whose second-order rate constant was characteristic of the particular substrate. This pH-dependent phenomenon of hysteresis on the part of myeloblastin, that is not manifested by the closely related leucocyte elastase, may have a physiological control function during phagocytosis by damping the rate of interconversion between enzymically inactive and active enzyme conformations.

INTRODUCTION

Human myeloblastin was originally described as proteinase 3, the 'third' serine endopeptidase from the azurophil granules of human polymorphonuclear leucocytes besides elastase and cathepsin G [1]. The purification of the enzyme [2] and subsequent studies established that the human polymorphonuclear leucocyte proteins known as AGP7 (azurophil granule protein 7), p29b, myeloblastin and proteinase 4 [3–7] were the same enzyme originally described as proteinase 3. Following recent recommendations we use here the name myeloblastin [8,9]. While exploring the substrate and inhibitor specificity of myeloblastin we observed a remarkably slow gain of enzymic activity after rapidly increasing the pH from acidic to neutral values. This phenomenon, known from other classes of enzymes, appears to be new among the cellular endopeptidases. We describe below the kinetic properties of these findings in terms of a slow conformational transition that shares many properties with the concepts of 'hysteresis' developed by Frieden [10] and 'enzyme memory' described by Ricard et al. [11]. We also propose a possible physiological function of this hysteretic phenomenon as a means of controlling an unwanted transition of myeloblastin to a proteolytically active form during phagocytosis.

MATERIALS AND METHODS

Human leucocyte elastase (EC 3.4.21.37) was purified as previously described [12] and myeloblastin (EC 3.4.21.76) according to Kao et al. [2]. To preserve enzyme activity, soon after purification myeloblastin was stored frozen at $-20\text{ }^{\circ}\text{C}$ in a 0.1 M glycine/HCl, 0.1 M NaCl buffer, pH 3.2, and intermediate dilutions before assay were also done in this buffer. Kinetic measurements were performed at $25 \pm 1\text{ }^{\circ}\text{C}$ in 50 mM sodium/potassium phosphate buffer, pH 7.00 ± 0.02 , ionic strength 0.11 M, 0.05 % (w/v) Triton X-100. Substrates (Bachem, Bubendorf, Switzerland) were Suc-AAPV-NMec, MeO-Suc-AAPV-NMec, MeO-Suc-AAPV-pNA and MeO-Suc-K(Pic)APV-pNA [where Suc is

succinyl, AAPV is Ala-Ala-Pro-Val, -NMec is 7-(4-methyl)-coumarylamide, MeO-Suc is methoxysuccinyl, -pNA is 4-nitro-anilide and K(Pic)APV is Lys(2-picolinoyl)-Ala-Pro-Val] that were dissolved in DMSO before being diluted into the assay buffer to give a final concentration of 0.5 % (v/v) for the organic solvent. The active-site concentration of leucocyte elastase was determined by titration with MeO-Suc-AAPV-CH₂Cl and measurement of the residual activity with MeO-Suc-AAPV-pNA. The concentration of a freshly made up solution of 3,4-dichloroisocoumarin was determined by titration of a leucocyte elastase sample of known concentration and then myeloblastin was titrated with this 3,4-dichloroisocoumarin solution using MeO-Suc-AAPV-NMec as substrate to measure residual activity. Progress curves with the fluorogenic substrates (-NMec as the leaving group) were continuously monitored using an AMINCO SPF-500 spectrofluorimeter operating in the ratio mode. Excitation and emission wavelengths were set at 383 and 455 nm, respectively, and the recorder scale was calibrated with 7-amino-4-methylcoumarin solutions of known concentrations determined using an absorption coefficient of $16000\text{ M}^{-1}\cdot\text{cm}^{-1}$ at 342 nm. With -pNA as the leaving group reaction progress was monitored photometrically at 405 nm, absorption coefficient $9920\text{ M}^{-1}\cdot\text{cm}^{-1}$ [13]. Non-linear regression analysis of experimental data was performed with Fig.P (Biosoft, Cambridge, U.K.) and Minsq II (MicroMath, Salt Lake City, UT, U.S.A.) software.

THEORY

When myeloblastin was kept at pH 3.2 and rapidly diluted into the final assay buffer at pH 7.0 containing substrate, the progress curves of product concentration versus time were characterized by a 'concave-up' lag phase lasting several minutes, depending on the substrate concentration. Some possible mechanisms accounting for hysteretic enzyme activation upon a rapid change in pH were considered. The model must account for the following observed properties: (1) progress curves are biphasic, consisting of a concave-up region followed by a linear one, as in the case of

Abbreviations used: MeO-Suc, methoxysuccinyl; Suc, succinyl; -pNA, 4-nitroanilide; -NMec, 7-(4-methyl)coumarylamide; AAPV, Ala-Ala-Pro-Val; K(Pic)APV, Lys(2-picolinoyl)-Ala-Pro-Val.

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slow binding inhibition when reaction is started by adding substrate; (2) the apparent first-order activation rate constant depends linearly upon the concentration of substrate ($[S]$) and its plot versus $[S]$ has a positive intersection on the ordinate axis; (3) enzyme activation occurs also in the absence of substrate, but at a lower rate; (4) a substrate-dependent velocity at zero time is present. The minimal reaction scheme accounting for all the observed properties is shown below as Mechanism 1

Mechanism 1

where E_i = inactive enzyme, E = active enzyme, S = substrate and P = product(s). The presence of active enzyme at the beginning of the reaction is taken into account by the equilibrium between E_i and E , $[E_i]/[E] = k_{-5}/k_5 = K_5$, at acidic pH, where the subscripts 5 and -5 distinguish the constants at pH 3.2 from those at pH 7 (subscripts 3 and -3). Although E at acidic pH is also enzymically inactive because of an unfavourable pH for a serine endopeptidase, this form, contrary to E_i , will immediately be active on raising the pH. The following assumptions were made for deriving a rate constant for this mechanism: (1) steady-state conditions are set up rapidly between E , S and products; (2) the substrate concentration remains constant for the time during which reaction is monitored, $[S] \approx [S]_i$, i.e. there is no substrate depletion and product accumulation, a condition that could be controlled experimentally; (3) the steps governed by k_3 , k_{-3} and k_4 , k_{-4} are slow on the steady-state time scale; (4) the reaction is started by adding enzyme dissolved in an acidic buffer to a neutral buffer containing substrate. The integrated rate equation for Mechanism 1 (derivation in the Appendix) is given by:

$$[P] = v_s t + (v_z - v_s)(1 - e^{-\lambda t})/\lambda \quad (1)$$

where $[P]$ is the product concentration at a given time t , v_s is the reaction velocity after attainment of the steady state, v_z is the reaction velocity at time zero, and λ is a first-order rate constant describing the exponential phase preceding the steady state. Eqn. (1) is a general expression that equally well applies to hysteretic enzymes and to slow-binding modifiers [10,14]. A particular mechanism is characterized by the individual expression of λ , v_s and v_z . For Mechanism 1:

$$\lambda = k_3 + k_4[S] + \frac{k_{-3}K_m + k_{-4}[S]}{K_m + [S]} \quad (2)$$

$$v_s = \frac{V[S]}{K_m + [S] + \frac{k_{-3}K_m + k_{-4}[S]}{k_3 + k_4[S]}} \quad (3)$$

$$v_z = \frac{V[S]}{(K_m + [S])(1 + K_5)} \quad (4)$$

These expressions mean that, when adding enzyme stored at acidic pH to the assay buffer at neutral pH containing substrate,

there will be a portion of enzyme present as E_i and another portion present as E , the distribution between the two forms being dictated by K_5 , the equilibrium constant between E_i and E at acidic pH. The active enzyme will immediately give rise to ES , so that, at $t = 0$, $v_z > 0$. Soon after adding enzyme to the assay buffer, the pH will be neutral and the exponential approach to the steady state will be governed by the values of k_3 and $k_4[S]$.

RESULTS

For the verification of Mechanism 1 four substrates were tested, Suc-AAPV-NMec, MeO-Suc-AAPV-NMec, MeO-Suc-AAPV-pNA and MeO-Suc-K(Pic)APV-pNA, but the tetrapeptide Suc-AAPV-NMec (k_{cat} and K_m in Figure 2B) was selected for the final experiments because it fulfilled the criterion $[S] \approx [S]_i$ during the whole course of the reaction. With other substrates, having higher k_{cat} values, the amount of product after 10–20 min of reaction was too high for this condition to be valid. The progress curves obtained at various substrate concentrations revealed that no more than 0.1 % of the available substrate was converted into product (Figure 1). Eqn. (1) was fitted to the primary data by non-linear regression to give a set of v_z , v_s and λ values, together with their standard errors, for each substrate concentration. The dependence of these parameters upon $[S]$ was then examined (Figure 2). Although a hyperbolic dependence of λ upon $[S]$ should be expected from eqn. (2), statistical analysis suggested that a straight line fitted the data better than a hyperbola. This is possible if $(k_{-3}K_m + k_{-4}[S]) \ll (K_m + [S])$ so that eqn. (2) reduces to eqn. (5):

$$\lambda = k_3 + k_4[S] \quad (5)$$

In agreement with eqns. (3) and (4), plots of v_s and v_z versus $[S]$ were hyperbolic (Figures 2B and 2C). Too many parameters are, however, present in eqns. (3) and (4) for a meaningful regression analysis, but a further simplification can be inferred from the dependence of the v_s/v_z ratio upon substrate concentration (eqn. 6).

$$\frac{v_s}{v_z} = \frac{(K_m + [S])(1 + K_5)}{K_m + [S] + \frac{k_{-3}K_m + k_{-4}[S]}{k_3 + k_4[S]}} \quad (6)$$

Figure 1 Progress curves for myeloblastin activation

For clarity only five curves with a few experimental points sampled from the continuously recorded progress curves are shown. Solid lines were drawn with the best-fit parameters according to eqn. (1). The substrate was Suc-AAPV-NMec, at the final concentrations shown near the curves, in 50 mM sodium/potassium phosphate buffer, ionic strength 0.11 M, 0.05 % (w/v) Triton X-100, 0.5 % DMSO, pH 7.00, temperature 25 ± 1 °C. Reactions were started by adding myeloblastin from a pH 3.2 storage buffer (final concentration 30 nM of titrated active sites) into the substrate solution.

Figure 2 Calculation of the kinetic parameters

Best-fit values of λ , v_s and v_z from Figure 1 were analysed by weighted linear or non-linear regression using $1/\text{S.E.M.}^2$ as the individual weighting factors (bars = S.E.M.; some S.E.M. values lie within the symbol size). The equations used were: eqn. (5) (A), eqn. (7) (B) and eqn. (4) (C). The relevant best-fit parameters are shown in the respective panels.

v_s/v_z is a hyperbolic function of $[S]$, increasing between $(1 + K_5)/(1 + k_{-3}/k_3)$ for $[S] \rightarrow 0$ and $(1 + K_5)$ for $[S] \rightarrow \infty$. However, the v_s/v_z ratio was found to be constant in the substrate concentration range examined, and this is only possible if $(k_{-3}K_m + k_{-4}[S]) \ll (k_3 + k_4[S])$. Under these conditions, eqn. (3) reduces to the Henri-Michaelis-Menten equation (eqn. 7):

$$v_s = V[S]/(K_m + [S]) \quad (7)$$

The fact that v_s/v_z remains constant at any $[S]$ also shows that $k_{-3} \ll k_3$, meaning that the enzyme is present as a single, fully active form at neutral pH. Thus, the constant v_s/v_z ratio equals $(1 + K_5)$. The mean $\pm$ S.D. of all v_s/v_z values calculated from Figures 2(B) and 2(C) was 4.7 ± 0.2 , i.e. $K_5 = 3.7$. For these reasons, eqn. (5) was fitted by weighted linear regression to the data in Figure 2(A), and eqns. (7) and (4) by weighted non-linear regression to those in Figures 2(B) and 2(C), respectively. The weighting factors were the individual $1/\text{S.E.M.}^2$ values from

analysis of the primary data. This was necessary because, for increasing $[S]$, the errors of λ and v_z also increased considerably, while the errors for v_s were smaller. The ordinate intercept in Figure 2(A) represents the sum $k_3 + k_{-3}$ (eqn. 2). Since $k_{-3} \ll k_3$ (see above), it corresponds to k_3 , the activation rate constant in the absence of substrate, $(3.3 \pm 0.1) \times 10^{-3} \text{ s}^{-1}$. From the slope of λ versus $[S]$ the substrate-dependent second-order rate constant k_4 was calculated as $2.2 \pm 0.1 \text{ M}^{-1} \cdot \text{s}^{-1}$. Figure 2(B) supplied k_{cat} and K_m , while the fit of eqn. (4) to the v_z data provided an estimate of the equilibrium constant K_5 (Figure 2C). Since eqn. (4) would require a fit with three parameters, it would result in meaningless estimates with the few data points available. Therefore, V and K_m determined from Figure 2(B) were considered known constants in eqn. (4) to yield $K_5 = 3.75$, in agreement with the value calculated above from the v_s/v_z ratio. The relevant kinetic parameters mentioned above are shown in the three panels of Figure 2.

The following additional experiments, not shown here, were performed as described above for Suc-AAPV-NMec. For the related substrate MeO-Suc-AAPV-NMec k_3 and k_4 were about $3 \times 10^{-3} \text{ s}^{-1}$ and $17 \text{ M}^{-1} \cdot \text{s}^{-1}$, respectively. With MeO-Suc-AAPV-pNA and MeO-Suc-K(Pic)APV-pNA, the approach to the steady state was much faster. Although with these substrates the exponential phase of the reaction could not be measured with sufficient accuracy for calculating k_3 and k_4 , the experiments revealed that the rate constant k_4 is a characteristic of the particular substrate. Human leucocyte elastase was transferred to the same buffer at pH 3.2 used for myeloblastin and incubated for 15 h. When rapidly diluted from this low-pH buffer into the assay buffer at pH 7.0 in the presence of substrates, elastase manifested from the very beginning of the reaction a linear steady-state rate. Another experiment consisted of incubating myeloblastin and elastase for 15 h at pH 3.2 in the presence of Suc-AAPV-NMec ($[S]_i = 5K_m$, $[E]_i = 800 \text{ nM}$). After diluting the mixtures into the assay buffer at pH 7.0, the progress curves were linear for elastase and concave-up for myeloblastin and coincided with those obtained by adding the enzymes from an acidic storage buffer to the substrate solution at pH 7.0 without preincubation.

DISCUSSION

The slow kinetic response of myeloblastin to a pH jump shares many properties in common with the concepts described as 'hysteresis' [10] and as 'enzyme memory' [11] (reviewed in refs. [15–17]). However, an explicit mathematical derivation of the equations describing Mechanism 1 for myeloblastin has not been reported previously. The hysteretic behaviour of myeloblastin, characterized by a slow gain of full activity when rapidly increasing the pH from 3.2 to 7.0, was not shared by the closely related enzyme leucocyte elastase. While the low pH of 3.2 was empirically chosen with the purpose of stabilizing enzyme activity, the same phenomenon was also observed when raising the pH from 4.0–4.5 to 7.0. The kinetic mechanism of the process could be described in terms of a spontaneous first-order and of a substrate-dependent second-order path of activation at neutral pH. Substrates were not able to induce the $E_i \rightarrow E$ or $E_i \rightarrow ES$ transition at acidic pH in myeloblastin and, in the case of elastase, the enzyme either retained its fully active form at acidic pH, or its equilibration with any inactive form was very fast. In the absence of substrates, myeloblastin was activated with $t_{1/2} = 210 \text{ s}$, i.e. activity was almost completely gained (99.2% after $7 \times t_{1/2}$) after 24.5 min. In the presence of substrates activation occurred faster and the rate contained the contributions both of the spontaneous and of the substrate-dependent process, e.g.

with 2.5 mM Suc-AAPV-NMec, $t_{1/2} = 79$ s, i.e. 99.2 % of activity was gained after 9.2 min. With substrates having high specificity constants, such as MeO-Suc-K(Pic)APV-pNA, the approach to the steady state was attained very quickly and, at high substrate concentrations, was barely observable using conventional spectrophotometric methods.

Although kinetics alone is not sufficient to explain the molecular basis for the hysteretic properties of myeloblastin, it can be proposed that E and E_i in Mechanism 1 represent two different conformations of the enzyme. The equilibrium constant $K_5 = 3.7$ between E and E_i means that, at pH 3.2, 79 % of myeloblastin is present in the inactive conformation E_i and 21 % in the active conformation E. Ionization or protonation of strategic ionizable amino acid side chains is responsible for 'slow' conformational changes that can be observed kinetically and may manifest co-operative effects even in monomeric enzymes [18]. Examples are the chloroplast fructose 1,6-bisphosphatase [19], the soybean β -glucosyltransferase [20] and the sea-urchin ovoperoxidase [21]. In the case of oligomeric enzymes, pH-dependent hysteresis has also been interpreted in terms of reversible association/dissociation of subunits such as the dimer-tetramer transition of bovine liver phosphofructokinase [22]. The phenomenon of pH-induced slow conformational transition is less common among the endopeptidases and the case of myeloblastin may be just the second example after that observed under artificial conditions, and apparently without physiological function, for chymotrypsin [23].

Whether the hysteretic behaviour of myeloblastin has or has not a physiological significance remains at present a matter of speculation. The following concept is proposed as a working hypothesis. During phagocytosis, the pH within the phagocytic vacuoles of the polymorphonuclear neutrophilic leucocyte is subjected to temporal changes in an apparently species-dependent manner [24–26]. In man, 5 min after initiation of phagocytosis, the pH of the phagolysosome is 7.8; it falls to 7.4 after 15 min, to 6.4 after 30 min and 5.7 after 60 min (approximate values from ref. [25]). In the rat, after a first rapid rise near to neutral, the intravacuolar pH drops to 6.5 after 3 min and further to approx. 4 within 7–15 min [24]. A proposed physiological function for myeloblastin is its bactericidal activity, a property shared with other proteins of the azurophil granules, cathepsin G, elastase and azurocidin, collectively termed 'serprocidins' [5], and other polypeptides of the neutrophils [27–29]. The different bactericidal proteins of the polymorphonuclear leucocytes work synergistically and manifest preferential ionic strength and pH conditions for optimal activity. It has been proposed that the antibacterial properties of these proteins may find their optimal conditions at some point in the history of the phagolysosome [30]. The bactericidal activity of myeloblastin, cathepsin G and elastase is independent of proteolytic activity [4,31], with a few exceptions for cathepsin G and elastase [32,33]. Azurocidin, although sharing considerable sequence similarity with elastase and cathepsin G and possessing a potentially functional active centre as a serine endopeptidase, is enzymically inactive because it contains glycine instead of serine at the expected catalytic site [7]. Considering the proteolytic activity of the serprocidins, it has been demonstrated that only elastase, but not cathepsin G and myeloblastin, is able to activate the latent forms of antibiotic proteins of the bactericidal family, stored in the large granules of the bovine neutrophils and which become exposed to serprocidins

during phagocytosis [34]. It is possible that the proteolytic activity of myeloblastin is not only not needed, but even unwanted, during the phagocytic event. Moreover, since the optimum for myeloblastin bactericidal activity is at pH 5.5 [35], the protein must probably, as far as possible, retain a particular conformation during the excursion of pH from acidic to neutral and back during phagocytosis. The hysteretic behaviour would allow such a property to be satisfied through 'damping' of an unwanted conformational change and the concomitant rise of proteolytic activity. The rate constants for myeloblastin's hysteretic transition fall within the time-range of pH changes during phagocytosis described above.

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APPENDIX

Derivation of the equations for Mechanism 1

The characteristics of Mechanism 1 and the assumptions made were described in the Theory section. For Mechanism 1, the differential equation for $[E_i]$, the steady-state condition for $[ES]$ and the conservation equation for the enzyme are:

$$d[E_i]/dt = k_{-3}[E] + k_{-4}[ES] - (k_3 + k_4[S])[E_i] \quad (A1)$$

$$[E][S]/[ES] = K_m \quad (A2)$$

$$[E] + [ES] + [E_i] = [E]_t \quad (A3)$$

Combining eqns. (A2) and (A3) we obtain:

$$[E] = \frac{[E]_t - [E_i]}{1 + [S]/K_m} \quad (A4)$$

$$[ES] = \frac{[E]_t - [E_i]}{1 + K_m/[S]} \quad (A5)$$

Introducing eqns. (A4) and (A5) into (A1) we get:

$$\begin{aligned} \frac{d[E_i]}{dt} = [E]_t & \left(\frac{k_{-3} + k_{-4}[S]/K_m}{1 + [S]/K_m} \right) \\ & - [E_i] \left(k_3 + k_4[S] + \frac{k_{-3} + k_{-4}[S]/K_m}{1 + [S]/K_m} \right) \end{aligned} \quad (A6)$$

Eqn. (A6) is a first-order linear equation of the form:

$$d[E_i]/dt = A - \lambda[E_i] \quad (A7)$$

where

$$A = [E]_t \left(\frac{k_{-3}K_m + k_{-4}[S]}{K_m + [S]} \right) \quad (A8)$$

and λ is given by text eqn. (2). Thus, eqn. (A6) can be rewritten as:

$$-\frac{1}{\lambda} \frac{d\{A - \lambda[E_i]\}}{dt} = A - \lambda[E_i] \text{ or } \frac{dy}{y} = -\lambda dt \quad (A9)$$

where $y = A - \lambda[E_i]$. Integration yields:

$$\ln(y/y_0) = -\lambda t \text{ or } y = y_0 e^{-\lambda t} \quad (A10)$$

The integration constant y_0 can be calculated from the boundary condition that, at $t = 0$, the only equilibrium is between E_i and E according to the equilibrium constant K_5 , namely $[E_i]/[E] = K_5$, so that $[E]_t = [E_i] + [E] = [E_i](1 + 1/K_5)$ and $[E_i] = \rho[E]_t$, with $\rho = K_5/(1 + K_5)$.

$$y_0 = y|_{t=0} = A - \lambda[E_i]_{t=0} = A - \lambda[E]_t \frac{K_5}{1 + K_5} = A - \rho\lambda[E]_t \quad (A11)$$

Taking into account the significance of y , from eqn. (A10) we obtain:

$$[E_i] = 1/\lambda(A - y_0 e^{-\lambda t}) \quad (A12)$$

Substitution of the expression for $[E_i]$ into eqn. (A5) yields:

$$[ES] = \frac{[E]_t}{1 + K_m/[S]} - \frac{A}{\lambda(1 + K_m/[S])} + \frac{y_0 e^{-\lambda t}}{\lambda(1 + K_m/[S])} \quad (A13)$$

From the expression of the rate equation, $v = k_2[ES]$, and eqn. (A11), after some rearrangement:

$$v = \frac{k_2[E]_t[S]}{K_m + [S]} \left\{ 1 - \frac{A}{\lambda[E]_t} - \left(\rho - \frac{A}{\lambda[E]_t} \right) e^{-\lambda t} \right\} \quad (A14)$$

or

$$v = \frac{k_2[E]_t[S]}{K_m + [S]} [1 - \sigma - (\rho - \sigma)e^{-\lambda t}] \quad (A15)$$

with

$$\sigma = A/\lambda[E]_t$$

The reaction velocities at steady state (v_s) and at zero time (v_z) are obtained from eqn. (A15) for $t = \infty$ and $t = 0$ to give text eqns. (3) and (4), respectively. With the aid of these two equations the rate equation can be rewritten as:

$$v = v_s + (v_z - v_s)e^{-\lambda t} \quad (A16)$$

that can be integrated to give:

$$[P] = v_s t - \left(\frac{v_z - v_s}{\lambda} \right) e^{-\lambda t} + C \quad (A17)$$

The integration constant C is calculated from the boundary condition $[P] = 0$ for $t = 0$, namely $C = (v_z - v_s)/\lambda$. Insertion of this value in eqn. (A17) gives text eqn. (1).