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BINDING OF THE RECOMBINANT PROTEINASE INHIBITOR EGLIN c FROM LEECH *HIRUDO MEDICINALIS* TO HUMAN LEUKOCYTE ELASTASE, BOVINE α-CHYMOTRYPSIN AND SUBTILISIN CARLSBERG: THERMODYNAMIC STUDY

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The effect of pH and temperature on the apparent association equilibrium constant (K_a) for the binding of the recombinant proteinase inhibitor eglin c from leech *Hirudo medicinalis* to human leukocyte elastase (EC 3.4.21.37), bovine α-chymotrypsin (EC 3.4.21.1) and subtilisin Carlsberg (EC 3.4.21.14) has been investigated. On lowering the pH from 9.5 to 4.5, values of K_a for eglin c binding to the serine proteinases considered decrease thus reflecting the acid-pK shift of the invariant histidyl catalytic residue (His57 in human leukocyte elastase and bovine α-chymotrypsin, and His64 in subtilisin Carlsberg) from $\simeq 6.9$, in the free enzymes, to $\simeq 5.1$, in the enzyme:inhibitor adducts. At pH 8.0, values of the apparent thermodynamic parameters for eglin c binding are: human leukocyte elastase – $K_a = 1.0 \times 10^{10}\,M^{-1}$, $\Delta G^o = -13.4$ kcal/mol, $\Delta H^o = +1.8$ kcal/mol, and $\Delta S^o = +52$ entropy units; bovine α-chymotrypsin – $K_a = 5.0 \times 10^9\,M^{-1}$, $\Delta G^o = -13.0$ kcal/mol, $\Delta H^o = +2.0$ kcal/mol, and $\Delta S^o = +51$ entropy units; and subtilisin Carlsberg – $K_a = 6.6 \times 10^9\,M^{-1}$, $\Delta G^o = -13.1$ kcal/mol, $\Delta H^o = +2.0$ kcal/mol, and $\Delta S^o = +51$ entropy units (values of K_a, ΔG^o and ΔS^o were obtained at 21°C; values of ΔH^o were temperature independent over the range explored, i.e. between 10°C and 40°C; 1 kcal = 4184 J).

Thermodynamics of eglin c binding to the serine proteinases considered has been analyzed in parallel with those of related (pro)enzyme:macromolecular inhibitor systems. Considering the known molecular models, the observed binding behaviour of eglin c was related to the inferred stereochemistry of the proteinase:inhibitor contact regions.

KEY WORDS: Recombinant proteinase inhibitor eglin c (from leech *Hirudo medicinalis*), human leukocyte elastase, bovine α-chymotrypsin, subtilisin Carlsberg, enzyme:inhibitor adduct formation, thermodynamics (of enzyme:inhibitor complex formation), pH effects (on enzyme:inhibitor adduct formation).

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INTRODUCTION

Eglins, small proteinase inhibitors present in the leech *Hirudo medicinalis*, are members of the potato inhibitor family.[1-3]

Two isoinhibitors (eglin b and c) are known, showing indistinguishable inhibition parameters in their reaction with various proteinases.[4] These macromolecules consist of a single polypeptide chain of 70 amino acid residues[5] with a unique replacement at position 35 (His in eglin b *versus* Tyr in eglin c).[4] The X-ray structures of the complexes of subtilisin Carlsberg with the native and the recombinant inhibitor eglin c have been solved at atomic resolution.[6,7] Recently, the recombinant eglin genes have been synthesized and expressed in *Escherichia coli*.[8]

Because of their specificity, efficiency or potency, low toxicity and "bioavailability",[9] eglins have a therapeutic potential in the pathogenesis of pulmonary diseases and inflammatory processes[9-12] involving leukocyte elastase and cathepsin G, considered as the main inhibitor target-enzymes. Moreover, inhibition of chymotrypsin and subtilisin by eglins[1-3] is probably of little pharmacological consequence.[9]

In order to shed more light on inhibitory properties, thermodynamics of binding of the recombinant eglin c to human leukocyte elastase, bovine α-chymotrypsin and subtilisin Carlsberg has been determined between pH 4.5 and 9.5, and from 10°C and 40°C. These results have been analyzed in parallel with the molecular and binding properties of related serine (pro)enzyme:macromolecular inhibitor systems.

MATERIAL AND METHODS

Materials

Human leukocyte elastase (from purulent sputum) was purchased from Elastin products Co. (Pacific, U.S.A.). Bovine α-chymotrypsin, treated with *N*-α-*p*-tosyl-L-lysine chloromethyl ketone in order to abolish tryptic activity, and subtilisin Carlsberg were obtained from Sigma Chemical Co. (St. Louis, U.S.A.). Purified recombinant eglin c (batch no. 32.968 – 800685) was prepared by Dr. H.P. Walliser (Ciba-Geigy AG, Basel, CH). Methoxysuccinyl-L-alanyl-L-alanyl-L-prolyl-L-valine *p*-nitroanilide, *N*-α-carbobenzoxy-L-tyrosine *p*-nitrophenyl ester and azocasein were obtained from Sigma Chemical Co. (St. Louis, U.S.A.). All the other reagents were from Merck AG (Darmstadt, F.R.G.). All chemicals were of analytical grade and used without further purification. The characterization of human leukocyte elastase, bovine α-chymotrypsin, subtilisin Carlsberg, eglin c, methoxysuccinyl-L-alanyl-L-alanyl-L-prolyl-L-valine *p*-nitroanilide, *N*-α-carbobenzoxy-L-tyrosine *p*-nitrophenyl ester and azocasein has been reported elsewhere.[1-3,13-16]

Determination of the Apparent Enzyme:Inhibitor Association Equilibrium Constant (K_a)

Values of the apparent association equilibrium constant (K_a) for the enzyme:inhibitor adduct formation were determined, between pH 4.5 and 9.5 (I = 0.1 M) and from 10 (±0.5)°C to 40(±0.5)°C, by the evaluation of the inhibitory effect of eglin c on the hydrolysis of methoxysuccinyl-L-alanyl-L-alanyl-L-prolyl-L-valine *p*-nitroanilide, *N*-α-carbobenzoxy-L-tyrosine *p*-nitrophenyl ester and azocasein catalyzed by human leukocyte elastase,[16] bovine α-chymotrypsin[15] and subtilisin Carlsberg,[1] respectively.

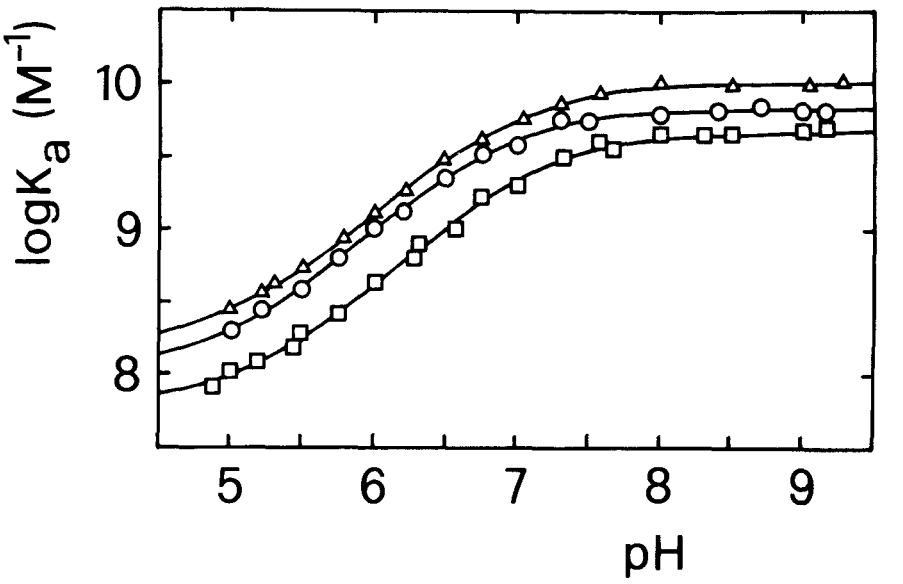

FIGURE 1 pH dependence of the apparent association equilibrium constant (K_a) for eglin c binding to human leukocyte elastase (△), bovine α-chymotrypsin (□) and subtilisin Carlsberg (○) at 21°C. Unbroken lines, calculated from eqn. (1) with the following sets of parameters: human leukocyte elastase — C = 10.0, pK_{UNL} = 6.9, and pK_{LIG} = 5.1; bovine α-chymotrypsin — C = 9.70; pK_{UNL} = 7.1, and pK_{LIG} = 5.2; and subtilisin Carlsberg — C = 9.82, pK_{UNL} = 6.8, and pK_{LIG} = 5.0, were obtained with an iterative non-linear least-squares curve fitting program. An average error value of ±12% was evaluated for K_{UNL} and K_{LIG} values, as the standard deviation, according to the curve fitting procedure. The pH profile was explored using the following buffers: acetate (pH 4.5 to 6.0); phosphate (pH 6.0 to 8.5); and borate/glycine (pH 8.5 to 9.5); all at I = 0.1 M (sodium salts). No specific ion effects were found using different buffers with overlapping pH values.

The biochemical procedure has been published previously.[1,15,16] An average error value of ±8% was ascribed to K_a values, as the standard deviation, according to the fitting procedure.[15]

RESULTS AND DISCUSSION

Under all the experimental conditions, eglin c binding to human leukocyte elastase, bovine α-chymotrypsin and subtilisin Carlsberg conforms to a simple equilibrium as indicated by the Hill coefficient (n) always being equal to 1.00 ± 0.03. Moreover, K_a values were always independent of the enzyme concentration.

The pH-dependent change in affinity of eglin c for human leukocyte elastase, bovine α-chymotrypsin and subtilisin Carlsberg, shown in Figure 1, is strictly reminiscent of those obtained for the binding of Kazal- and/or Kunitz-type inhibitors to bovine α- and/or β-trypsin, bovine trypsinogen, bovine α-chymotrypsin, porcine pancreatic β-kallikreins A and B, human urinary kallikrein and human Lys^{77}-plasmin,[15,17–20] and thus may be described with the same model. Therefore, the simplest mechanism (i.e., with the fewest ionization(s)) accounting for the observed data implies that lowering pH from 9.5 to 4.5, the decrease in K_a values (i.e., in affinity) for eglin c binding to

the serine proteinases considered reflects the acidic perturbation of a single ionizing group upon inhibitor association. According to linkage relations, this model leads to the following expression:

$$\log K_a = C - \log\{([H^+] + 10^{-pK_{UNL}})/([H^+] + 10^{-pK_{LIG}})\} - |\Delta pK|, \quad (1)$$

where C is a constant that corresponds to the alkaline asymptote of $\log K_a$, and pK_{UNL} and pK_{LIG} are the pK values of the apparent proton dissociation constants for the inhibitor-free (K_{UNL}) and inhibitor-bound (K_{LIG}) enzymes, respectively. Eqn. (1) has been used to generate the unbroken lines shown in Figure 1; the agreement with the experimental data is satisfactory.

In principle, the observed pH effects can be explained as a result of the pK perturbation of the functionally relevant group on the enzyme and/or the inhibitor. However, crystallographic data[6,7,21,22] suggest the assignment of the ionization to the enzyme side only. In fact: (i) on eglin c the only amino acid side chain likely to ionize between pH 4.5 and 9.5 is the P_1' residue (i.e., Asp46[23]) of the contact region, whose carboxylate is strongly involved in intramolecular hydrogen bonds and salt bridges supporting the reactive site, and thus cannot participate in bonding the enzyme; and (ii) the pH profiles for the binding of eglin c to the three proteinases considered are strongly similar to those published on the interaction of Kazal- and Kunitz-type inhibitors with serine (pro)enzymes, in which the only relevant ionizing group has been identified on the proteinase.[15,17–20]

Some idea about the chemical identity of the ionizable group controlling eglin c binding to human leukocyte elastase, bovine α-chymotrypsin and subtilisin Carlsberg may be gained from values of K_{UNL} and K_{LIG} (see Figure 1), which appear to be closely related to those calculated from the pH dependence of: (i) kinetics for the enzymatic hydrolysis of natural and synthetic substrates; as well as (ii) thermodynamics and kinetics for the binding of ligands and inhibitors.[13–15,26]

Inspection of the different active-site residues capable of affecting eglin c association suggests that the invariant histidyl residue, involved in the catalytic triad (i.e., His57 in human leukocyte elastase[22] and bovine α-chymotrypsin,[21] and His64 in subtilisin Carlsberg[6,7]) has a pK value comparable to the pK_{UNL} in free proteinases (see Figure 1). In this respect, the calculated pK shift of the one proton binding residue could be interpreted as reflecting the strengthening of the proteinase hydrogen bond between Ser221 OG and His64 NE2 in the subtilisin Carlsberg:eglin c adduct,[7] and possibly between Ser195 OG and His57 NE2 in the inhibitor complexes with human leukocyte elastase and bovine α-chymotrypsin. In analogy with serine (pro)enzyme:Kazal- and :Kunitz-type inhibitor systems, such hydrogen bond occurs upon adduct formation, being very weak or absent in the free (pro)enzymes (see Bolognesi *et al.*[27] for a review).

In order to gain information about what extent the magnitude of the apparent free energy change involved in the complex formation represents the apparent entropy and the apparent total heat effects, the influence of temperature on eglin c binding to human leukocyte elastase, bovine α-chymotrypsin and subtilisin Carlsberg has been investigated at pH 8.0;[28,29] Table I shows values of the relevant apparent thermodynamic parameters. Wherever the comparison was possible, the observed values were in good agreement with those obtained by different methods and taken from the literature,[1–3,9,16–27,30] if differences in the experimental conditions are considered.

Values of $\log K_a$ for eglin c binding to human leukocyte elastase, bovine α-chymotrypsin and subtilisin Carlsberg, determined between 10°C and 40°C, are linearly

TABLE I
Values of the apparent thermodynamic parameters for the binding of eglin c to human leukocyte elastase, bovine α-chymotrypsin and subtilisin Carlsberg (pH 8.0, phosphate buffer, $I = 0.1$ M)

Complex	K_a (M^{-1})	ΔG^0 (kcal/mol)	ΔH^0 (kcal/mol)	ΔS^0 (entropy units)
Human leukocyte elastase:eglin c	1.0×10^{10}	−13.4	+1.8	+52
Bovine α-chymotrypsin:eglin c	5.0×10^{9}	−13.0	+2.0	+51
Subtilisin Carlsberg:eglin c	6.6×10^{9}	−13.1	+2.0	+51

Values of K_a, ΔG^0 and ΔS^0 have been obtained at 21°C; values of ΔH^0 were determined between 10°C and 40°C (1 kcal = 4184 J). An average error value of ±8% was ascribed to K_a values, as the standard deviation (see the text). An average error value of ±12% was found for ΔH^0 values, as the standard deviation, according to the linear least-squares curve fitting procedure.

dependent (within experimental error) on $1/T$ (indicating apparent ΔC_p^0 values equal to 0) and allow the estimation of the apparent enthalpy variation associated with the enzyme:inhibitor adduct formation.[28,29] Both the apparent ΔH^0 and ΔS^0 (at 21°C) values[28,29] for eglin c binding to the proteinases considered are comparable to those reported for the association of Kazal- and Kunitz-type inhibitors to serine (pro)enzymes[18–20] and suggest that the adduct formation is an entropy-driven process. As proposed for other (pro)enzyme:inhibitor systems, the apparent positive ΔS^0 values could reflect the removal of the proteinase- and/or inhibitor-bound water molecules during complexation (see Bolognesi *et al.*[27] for a review).

As shown in Figure 1 and Table I, the apparent thermodynamic parameters for eglin c binding to human leukocyte elastase, bovine α-chymotrypsin and subtilisin Carlsberg are closely similar, in spite of different amino acids, contributed by the single proteinases considered,[7,21,22] involved in the enzyme:inhibitor contact area. This finding is in line with the view[20] that the apparent total binding energy in protein-protein interaction depends upon many energetically equivalent, possibly independent, contributions. In other words, in these complexes no interaction appears to be dominant and conformational changes induced by protein association are almost absent or very similar in the three adducts; otherwise, different flexibility in the recognition area(s) of human leukocyte elastase, bovine α-chymotrypsin and subtilisin Carlsberg would affect differently the apparent binding energy for eglin c association by taking advantage of diverse apparent entropy loss.

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28. Although in the literature values of thermodynamic parameters for serine (pro)enzyme:inhibitor adduct formation are usually calculated from graphs of logK (M^{-1} or M) on $1/T$,[1-4,17-20] they should be only taken as apparent estimates.[29] In fact, the application of the van't Hoff plot to a dimensioned equilibrium constant demands the use of relative concentrations, if a change in the mole number occurs in the reaction, and may imply oversimplified approximations.[29] Therefore, according to Keleti,[29] values of thermodynamic parameters quoted in the present study, and obtained from plots of $\log K_a$ (M^{-1}) on $1/T$, are nominated as apparent quantities.
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