J. Enzyme Inhibition, 1993, Vol. 7, pp. 57–64
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BINDING OF BOVINE BASIC PANCREATIC TRYPSIN INHIBITOR (KUNITZ) AS WELL AS BOVINE AND PORCINE PANCREATIC SECRETORY TRYPSIN INHIBITOR (KAZAL) TO HUMAN CATHEPSIN G: A KINETIC AND THERMODYNAMIC STUDY

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(Received July 12, 1992)

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Abbreviations used: cathepsin G; human cathepsin G; BPTI, bovine basic pancreatic trypsin inhibitor (Kunitz); bovine PSTI, bovine pancreatic secretory trypsin inhibitor (Kazal); porcine PSTI, porcine pancreatic secretory trypsin inhibitor (Kazal).

The effect of pH and temperature on kinetic and thermodynamic parameters for the binding of the bovine basic pancreatic trypsin inhibitor (Kunitz inhibitor; BPTI) as well as bovine and porcine pancreatic secretory trypsin inhibitor (Kazal inhibitor; bovine and porcine PSTI, respectively) to human cathepsin G (EC 3.4.21.20) has been investigated. The affinity of the macromolecular inhibitors examined for cathepsin G is characterized by an endothermic, entropy-driven, behaviour, and shows the following trend: BPTI > bovine PSTI > porcine PSTI. The affinity difference of BPTI as well as of bovine and porcine PSTI for cathepsin G is mostly accounted for by changes in the values of the apparent dissociation rate constant for the proteinase:inhibitor complex destabilization. On increasing the pH from 4.5 to 9.5 (at 25.0°C), the affinity of BPTI, as well as bovine and porcine PSTI for cathepsin G increases thus reflecting the acidic-p*K* shift of the His-57 catalytic residue from ≈ 6.9 in the free enzyme to ≈ 5.0 in the serine proteinase:inhibitor complexes. The BPTI as well as the bovine and porcine PSTI binding properties of cathepsin G have been analyzed in parallel with those of related serine (pro)enzyme/macromolecular inhibitor systems. Considering the known molecular models, the observed binding behaviour of BPTI as well as that of bovine and porcine PSTI to cathepsin G has been related to the inferred stereochemistry of the serine proteinase/inhibitor contact region(s).

KEY WORDS: Human cathepsin G; bovine basic pancreatic trypsin inhibitor (Kunitz); bovine pancreatic secretory trypsin inhibitor (Kazal); porcine pancreatic secretory trypsin inhibitor (Kazal); proteinase:inhibitor complex formation kinetics, thermodynamics, and pH and temperature effects.

INTRODUCTION

Human cathepsin G (3.4.21.20) is a serine proteinase, stored in the azurophyl granules of polymorphonuclear leukocytes, which, on the basis of its digestive activity toward several macromolecular substrates, could be involved in the pathogenesis of a wide variety of diseases. Thus, it has been suggested that cathepsin G may play a role in (*i*) the development of pulmonary emphysema and reumathoid arthritis, owing to its activity toward connective tissue proteins, (*ii*) the development of hypertension, based on its ability to convert angiotensinogen and angiotensin I to angiotensin II, (*iii*) muscle catabolism, catalyzing the cleavage of myosin and, (*iv*) the regulation of cell adhesion, cell mobility and cell migration, by selective hydrolysis of fibronectin (see Hörn and Heidland,[1] Virca *et al.*[2] and Cunningham and Long,[3] and references cited therein). The enzyme action may irreversibly be blocked by reaction with synthetic low molecular weight compounds such as aryl- and alkyl-sulfonyl fluorides, fluorophosphates, and chloromethyl ketones. Also, chymostatin and macromolecular protein proteinase inhibitors, such as eglin *c* (the serine proteinase inhibitor from leech *Hirudo medicinalis*), α_1-proteinase inhibitor, α_1-antichymotrypsin, α_2-macroglobulin, and inhibitors from soybean and limabean, have been reported to inactive cathepsin G. Strong (macro)molecular inhibitors of cathepsin G contain apolar residues (such as Leu, Met, Val, Phe or Tyr) at their P_1 and P'_2 positions (see Barrett and Salvesen,[4] Stein and Strimpler[5] and Brinkmann *et al.*[6] and references cited therein).

In the present communication the kinetics and thermodynamics for the binding of the bovine basic pancreatic trypsin inhibitor (Kunitz) as well as the bovine and porcine pancreatic secretory trypsin inhibitors (Kazal) to cathepsin G, determined between pH 4.5 and 9.5 as well as from 10.0°C to 40.0°C, are reported. The formation of the cathepsin G:BPTI, as well as :bovine and :porcine PSTI complexes has been analyzed in parallel with the molecular and binding properties of homologous serine (pro)enzyme/inhibitor systems.

MATERIALS AND METHODS

Materials

Cathepsin G was obtained from Elastin Products (Pacific, MO, USA) ad further purified as previously reported.[7] BPTI was kindly provided by Bayer AG (Wuppertal, FRG) and Lepetit S.p.A. (Milano, Italy), and further purified as detailed elsewhere.[8] Bovine and porcine PSTI (type I) were prepared as detailed elsewhere.[9-11] Methoxysuccinyl-L-alanine-L-alanine-L-proline-L-valine *p*-nitroanilide and methoxysuccinyl-L-alanine-L-alanine-L-proline-L-phenylalanine *p*-nitroanilide were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All the other products were from Merck AG (Darmstadt, FRG). All chemicals were of analytical grade and used without further purification. The characterization of cathepsin G, BPTI, bovine and porcine PSTI as well as methoxysuccinyl-L-alanine-L-alanine-L-proline-L-valine *p*-nitroanilide and methoxysuccinyl-L-alanine-L-alanine-L-proline-L-phenylalanine *p*-nitroanilide has been previously reported.[2,7-11]

Determination of the Apparent Kinetic and Thermodynamic Parameters for the Cathepsin G:Inhibitor Complex (De)stabilization

Values of the apparent second order rate constant (k_{on}) for inhibitor binding to cathepsin G were obtained, at pH 8.0 and 25.0°C, by the evaluation of the inhibitory effect of BPTI as well as that of bovine and porcine PSTI on the enzymatic hydrolysis of methoxysuccinyl-L-alanine-L-alanine-L-proline-L-valine *p*-nitroanilide and methoxysuccinyl-L-alanine-L-alanine-L-proline-L-phenylalanine *p*-nitroanilide, taking into account the simple competition of the inhibitors with the substrate for the proteinase.[12] Values of the apparent dissociation rate constant (k_{off}) for the destabilization of the cathepsin G:BPTI as well as :bovine and :porcine PSTI complexes were obtained, at pH 8.0 and 25.0°C, taking into account the replacement of cathepsin G by bovine β-trypsin within the enzyme:inhibitor complexes (i,e,. the recovery of the cathepsin G catalytic activity upon formation of the bovine β-trypsin:inhibitor complex).[13] Values of the apparent association equilibrium constant (K_a) for inhibitor binding to cathepsin G were obtained, between pH 4.5 and 9.5 and from 10.0°C to 40.0°C, by the evaluation of the inhibitory effect of BPTI as well as bovine and porcine PSTI on the enzymatic hydrolysis of methoxysuccinyl-L-alanine-L-alanine-L-proline-L-valine *p*-nitroanilide and methoxysuccinyl-L-alanine-L-alanine-L-proline-L-phenylalanine *p*-nitroanilide, taking into account the simple competition of the inhibitors with the substrate for the proteinase.[2,12,14] Values of K_a were also calculated, at pH 8.0 and 25.0°C, from values of k_{on} and k_{off} ($K_a = k_{on}/k_{off}$).[13] Values of the apparent free energy (δG°) for inhibitor binding to cathepsin G were calculated, at pH 8.0 and 25.0°C, from values of K_a.[14,15] Values of the apparent enthalpy variation (δH°) accompanying the enzyme:inhibitor complex formation were determined, at pH 8.0, from the linear dependence of log K_a on $1/T$ by the van't Hoff plot,[14,15] the temperature ranging between 10.0°C and 40.0°C. Values of the apparent entropy variation (δS°) for inhibitor binding to cathepsin G were calculated, at pH 8.0 and 25.0°C, from values of δG° and δH°.[14,15] A standard deviation of $\pm 8\%$ was evaluated for values of k_{on}, k_{off}, K_a (determined experimentally) and δG°, and of $\pm 12\%$ for values of K_a (calculated from values of k_{on} and k_{off}), δH° and δS°.[14]

RESULTS AND DISCUSSION

Under all experimental conditions, BPTI as well as bovine and porcine PSTI binding to cathepsin G conforms to a simple process, as indicated by the observation that over 95% of the time course for the serine proteinase:inhibitor complex (de)stabilization corresponds to a single exponential process. Furthermore, the binding isotherms for the enzyme:BPTI as well as :bovine and :porcine PSTI complex formation always display a Hill coefficient (n) equal to 1.00 ± 0.02. Next, values of K_a obtained experimentally are in excellent agreement with those calculated from values of k_{on} and k_{off} (see Table 1). Moreover, values of kinetic and thermodynamic parameters are always independent of the enzyme, inhibitor and substrate concentration.[16]

As shown in Figure 1 and Table 1, the affinity of the macromolecular inhibitors examined for cathepsin G shows the following trend: BPTI > bovine PSTI > porcine PSTI. Next, differences in k_{off} values for the proteinase:inhibitor complex destabilization mostly account for the affinity changes of BPTI as well as bovine and porcine PSTI binding, expressed by K_a values (see Table 1).

The higher affinity of BPTI for cathepsin G, as compared to bovine and porcine PSTI (see Table 1), may be related to the contained radius of the inhibitor reactive site (i.e., the "inhibitory head"), the distance between the C-α carbon atom of aminoacid residues at positions P_3 and P'_4 of the inhibitors a value considered being 11.6Å in BPTI and 20.0Å in porcine PSTI. In fact, upon BPTI binding, bovine trypsinogen adopts a structure that is almost coincident with that of the active enzyme, whereas porcine PSTI induces in the zymogen a novel conformation of the activation domain that differs from that of bovine β-trypsin and may be energetically less favourable.[17-23]

As previously reported for pancreatic secretory trypsin inhibitor binding to bovine (β-)trypsin(ogen), bovine α-chymotrypsin and human leukocyte elastase,[22] the affinity of bovine PSTI for cathepsin G is higher than that reported for porcine PSTI. This finding may reflect the direct influence on complex formation exerted by residues at sites P_8 and P_5, which host different amino acid residues in the two Kazal-type inhibitors (Asn in bovine PSTI *versus* Ser in porcine PSTI), and which are likely contributors to the intermolecular recognition events.[19,22]

In spite of the primary structure homology of cathepsin G with serine (pro)enzymes belonging to the chymotrypsin superfamily,[24] the affinity of BPTI as well as bovine and porcine PSTI for cathepsin G is at least six orders of magnitude lower than that observed for inhibitor binding to bovine β-trypsin (see Table 1 and Antonini *et al.*[17] for comparison). The different values of K_a for the cathepsin G: and bovine β-trypsin:inhibitor complex formation is in agreement with the well known different primary specificity properties displayed by these two homologous serine proteinases.[24] In particular, cathepsin G lacks Asp-189 (replaced by an alanyl residue) at the dead end of the primary specificity subsite (S_1)[24] which in the bovine (β-)trypsin(ogen):BPTI as well as :porcine PSTI adducts stabilizes the positively charged inhibitor side chain Lys-15 (in BPTI), and Lys.18 (in porcine PSTI).[19,23]

The affinity of BPTI as well as bovine and porcine PSTI for cathepsin G is similar to that observed for the inhibitor association to human leukocyte elastase, but lower by about two orders of magnitude than that reported for the formation of the bovine α-chymotrypsin:inhibitor complexes (see Table 1, Antonini *et al.*,[17] Fioretti *et al.*[20] and Ascenzi *et al.*[22] for comparison). These differences in K_a values for inhibitor binding to the chymotrypsin-like serine proteinases considered may be related to the structural

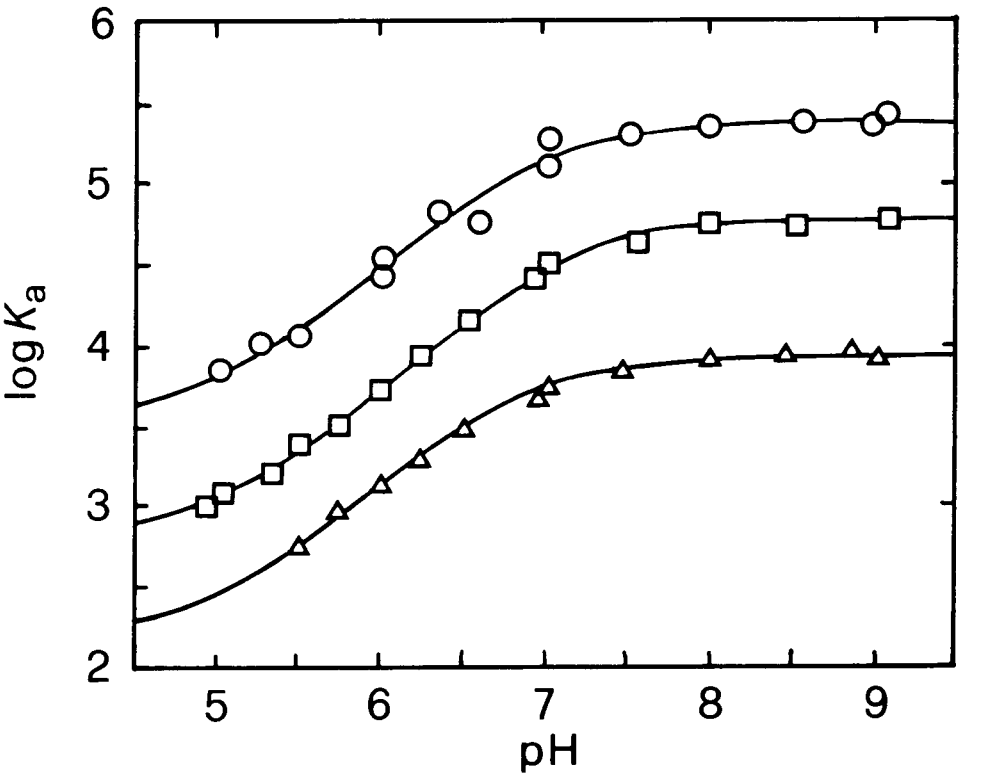

Figure 1 pH dependence of the apparent association equilibrium constant (K_a; M^{-1}) for BPTI (○) as well as bovine (□) and porcine (△) PSTI binding to cathepsin G at 25.0°C. The lines were generated from the following equation:[14,20]

$$\log K_a = C - \log\left\{\frac{([H^+] + 10^{-pK_{UNL}})}{([H^+] + 10^{-pK_{LIG}})}\right\} - \log\frac{10^{-pK_{LIG}}}{10^{-pK_{UNL}}}$$

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 equilibrium constants for the inhibitor-free (K_{UNL}) and the inhibitor-bound (K_{LIG}) serine proteinase, respectively. The lines, generated with the following sets of parameters: BPTI – $C = 5.4$, $pK_{UNL} = 6.9$, and $pK_{LIG} = 5.0$; bovine PSTI – $C = 4.8$, $pK_{UNL} = 7.1$, and $pK_{LIG} = 5.1$; and porcine PSTI – $C = 3.95$, $pK_{UNL} = 6.8$, and $pK_{LIG} = 5.0$, were obtained with an iterative non-linear least-squares curve fitting procedure which also allowed to ascribe a standard deviation of ±12% to 10^C, K_{UNL} and K_{LIG} values.[14] The pH profile was explored using the following buffers: acetate buffer, pH 4.5 to 6.0; phosphate buffer, pH 6.0 to 8.5; *N*-tris[hydroxymethyl]methylglycine/HCl buffer, pH 7.0 to 8.0; *N*-[2-hydroxyethylpiperazine]-N′-[2-ethanesulfonic acid] buffer, pH 7.5 to 8.5; borate/glycine buffer, pH 8.5 to 9.5; all 0.05 M to 0.2 M (sodium salts). No specific ion effects were found using different buffers with overlapping pH values. For further details, see text.

differences in the enzyme active center, in the surrounding loop regions, and to their varied interactions with BPTI as well as bovine and porcine PSTI. In particular, the proteinase regions 20–30, 45–54, 88–93, 139–152, 167–177, 190–198, 215–224 and 227–232 may be considered as potentially capable of steric hindrance on the incoming inhibitor in cathepsin G, in human leukocyte elastase and in bovine α-chymotrypsin because of their altered structural properties.[22,24–27]

Table 1 Values of apparent kinetic and thermodynamic parameters for BPTI as well as bovine and porcine PSTI binding to cathepsin G (pH 8.0)

Inhibitor	k_{on}[a,b] ($M^{-1} s^{-1}$)	k_{off}[a,b] (s^{-1})	K_a[a,b] (M^{-1})	$\delta G°$[a,b] (kJ mol^{-1})	$\delta H°$[a,c] kJ mol^{-1})	$\delta S°$[a,b] (J $mol^{-1} K^{-1}$)
BPTI	2.3×10^5	9.0×10^{-1}	2.5×10^5 2.6×10^5[d]	-3.1×10	$+1.8 \times 10$	$+1.6 \times 10^2$
Bovine PSTI	1.7×10^5	2.9	6.8×10^4 5.9×10^4[d]	-2.7×10	$+1.3 \times 10$	$+1.3 \times 10^2$
Porcine PSTI	1.3×10^5	1.6×10	8.1×10^3 8.1×10^3[d]	-2.2×10	$+1.4 \times 10$	$+1.2 \times 10^2$

[a]A standard deviation of $\pm 8\%$ was evaluated for k_{on}, k_{off}, K_a (obtained experimentally) and $\delta G°$ values, and of $\pm 12\%$ for K_a (calculated from k_{on} and k_{off} values), $\delta H°$ and $\delta S°$ values. For further details, see text.
[b]Values of k_{on}, k_{off}, K_a, $\delta G°$ and $\delta S°$ were obtained at 25.0°C. For further details, see text.
[c]Values of $\delta H°$ were obtained from the effect of temperature on values of K_a; the temperature ranged between 10.0°C and 40.0°C. For further details, see text.
[d]Values of K_a were calculated from values of k_{on} and k_{off}. For further details, see text.

Moreover, according to cathepsin G specificity (see Barrett and Salvesen,[4] Stein and Strimpler,[5] Brinkmann *et al.*[6] and references cited therein), it must be pointed out that recombinant BPTI homologues, showing a leucyl residue at position 15 (i.e., P_1; instead of Lys in the native inhibitor) and a phenylalanine or a tyrosyl side chain at position 17 (i.e., P'_2; instead of Arg in the native inhibitor), display K_a values of $\approx 1 \times 10^8 M^{-1}$ *versus* $2.5 \times 10^5 M^{-1}$ for wild-type BPTI (see Table 1 and Brinkmann *et al.*[6] for comparison).

Values of the apparent $\delta H°$ and $\delta S°$, given in Table 1, indicate that BPTI as well as bovine and porcine PSTI binding to cathepsin G is an endothermic, entropy-driven process.[21,22,28] The positive apparent values of $\delta S°$ (see Table 1) could reflect the increased degrees of freedom gained by the system when bound water molecules are removed from the serine proteinase and/or the inhibitor interacting surface(s) during complexation.[28]

The pH-dependent change in affinity (i.e., in K_a values) for BPTI as well as bovine and porcine PSTI binding to cathepsin G, shown in Figure 1, is strictly reminiscent of that observed for the binding of macromolecular inhibitors, (i.e., Kunitz- and Kazal-type inhibitors, eglin *c* and hirudin) to serine (pro)enzymes,[4,14,18,20–22,29,30] and thus may be described in identical terms. Therefore, the increase in the K_a values (i.e., in affinity) for BPTI as well as bovine and porcine PSTI association to cathepsin G on increasing the pH from 4.5 to 9.5, may be interpreted as reflecting the acidic p*K*-shift of the His-57 catalytic residue from ≈ 6.9 in the free enzyme to ≈ 5.0 in the serine proteinase:inhibitor complexes. The calculated acidic p*K*-shift of the His-57 catalytic residue could reflect, (*i*) the burial of the enzyme active site residues upon the serine proteinase:inhibitor complex formation, with change in the local dielectric constant, and, (*ii*) the concomitant strengthening of the cathepsin G Ser-195 OG—His-57 NE2 hydrogen bond, upon BPTI as well as bovine and porcine PSTI binding, which is very weak or absent in the inhibitor free proteinase.[4,19,23,26]

The reported data indicate that the different kinetic and thermodynamic parameters for the binding of macromolecular inhibitors (i.e., BPTI as well as the homologous bovine and porcine PSTI) to homologous serine proteinases belonging to the chymotrypsin superfamily (i.e., cathepsin G, bovine β-trypsin, bovine α-chymotrypsin and human leukocyte elastase) can vary substantially as a result of contained structural

perturbations occurring at the interacting surface(s), which alter specific recognition contact(s), but leave the inhibitor polypeptide backbone structure (i.e., the molecular scaffold) essentially unmodified.

Finally, from the physiopathological viewpoint, the reported data obtained *in vitro* (see Figure 1 and Table 1) together with the normal (plasma) level of the BPTI-like proteinase inhibitors ($\approx 1 \times 10^{-8}$ M),[31] as well as that of the therapeutic concentration of BPTI ($\approx 1 \times 10^{-6}$ M),[4] do not favour the hypothesis of any interaction of cathepsin G with BPTI and BPTI-like inhibitors. On the other hand, such a behaviour appears to be of interest in relation to a rationale for the therapeutic action of BPTI.

Acknowledgements

The authors want to dedicate this manuscript to Prof. Eraldo Antonini, who has greatly contributed to their own cultural formation, in the occasion of the 10th anniversary of his death on March 19 1983. We wish to thank Prof. Gino Amiconi for helpful discussions. This study was partially supported by grants from the Italian Ministry of University, Scientific Research and Technology (MURST), as well as from the Italian National Research Council (CNR), target oriented project Biotechnologie e Biostrumentazione.

References

1. Hörn, H. and Heidland, A. (eds.) (1982) *Proteases: Potential Role in Health and Diseases.* Plenum Press: New York.
2. Virca, G.D., Metz, G. and Schnebli, H.P (1984) *Eur. J. Biochem.*, **144**, 1–9.
3. Cunningham, D.D. and Long, G.L. (eds) (1987) *Proteinases in Biological Control and Biotechnology.* Alan R. Liss Inc.: New York.
4. Barrett, A.J. and Salvesen, G. (eds) (1986) *Proteinase Inhibitors.* Elsevier: Amsterdam.
5. Stein, R.L. and Strimpler, A.M. (1987) *Biochemistry*, **26**, 2611–2615.
6. Brinkmann, T., Schnierer, S. and Tschesche, H. (1991) *Eur. J. Biochem.*, **202**, 95–99.
7. Heck, L.W., Rostand, K.S., Hunter, F.A. and Bhown, A. (1986) *Anal. Biochem.*, **158**, 217–227.
8. Kassel, B. (1970) *Meth. Enzymol.*, **19**, 844–852.
9. Burk, P.J. (1970) *Meth. Enzymol.*, **19**, 906–914.
10. Schneider, S.L. and Laskowski, Sr., M. (1974) *J. Biol. Chem.*, **249**, 2009–2015.
11. Menegatti, E., Bortolotti, F., Minchiotti, L. and De Marco, A. (1982) *Biochim. Biophys. Acta.*, **707**, 50–58.
12. Ascenzi, P., Coletta, M., Amiconi, G., De Cristofaro, R., Bolognesi, M., Guarneri, M. and Menegatti, E. (1988) *Biochim. Biophys. Acta*, **956**, 156–161.
13. Vincent, J.-P. and Lazdunski, M. (1973) *Eur. J. Biochem.*, **38**, 365–372.
14. Ascenzi, P., Coletta, M., Amiconi, G., Bolognesi, M., Menegatti, E. and Guarneri, M. (1990) *Biol. Chem. Hoppe Seyler*, **371**, 389–393.
15. Keleti, T. (1983) *Biochem. J.*, **209**, 277–280.
16. Brinkmann *et al.*[6] overlooked cathepsin G inhibition by BPTI. In the absence of detailed experimental informations, we can only guess that the observed differences might be due to the effect of substrate concentration on BPTI binding to cathepsin G, in view of the low enzyme:inhibitor affinity and of the fast rates for binary complex (de)stabilization.
17. Antonini, E., Ascenzi, P., Bolognesi, M., Gatti, G., Guarneri, M. and Menegatti, E. (1983) *J. Mol. Biol.*, **165**, 543–558.
18. Amiconi, G., Ascenzi, P., Bolognesi, M., Menegatti, E. and Guarneri, M. (1988) In *Macromolecular Biorecognition: Principles and Methods*, (eds I.M. Chaiken, E. Chiancone, A. Fontata and P. Neri) pp. 117–130; Humana Press: Clifton.
19. Bolognesi, M., Ascenzi, P., Amiconi, G., menegatti, E. and Guarneri, M. (1988) In *Macromolecular Biorecognition: Principles and Methods*, (eds I.M. Chaiken, E. Chiancone, A. Fontana and P. Neri) pp. 81–100; The Humana Press: Clifton.
20. Fioretti, E., Angeletti, M., Passeri, D. and Ascoli, F. (1989) *J. Protein Chem.*, **8**, 51–60.
21. Fioretti, E., Angeletti, M., Cottini, M.T. and Ascoli, F. (1989) *J. Mol. Recogn.*, **2**, 142–146.

22. Ascenzi, P., Amiconi, G., Bolognesi, M., Onesti, S., Petruzzelli, R. and Menegatti, E. (1991) *J. Enz. Inhibit.*, **5**, 207–213.
23. Bode, W. and Huber, R. (1992) *Eur. J. Biochem.*, **204**, 433–451.
24. Greer, J. (1990) *Proteins* **7**, 317–334.
25. Tsukada, H. and Blow, D.M. (1985) *J. Mol. Biol.*, **184**, 703–711.
26. Bode, W., Wei, A.Z., Huber, R., Meyer, E., Travis, J. and Neumann, S. (1986) *EMBO J.*, **5**, 2453–2458.
27. Creighton, T.E. and Darby, N.J. (1989) *Trends Biochem. Sci.*, **14**, 319–324.
28. Amiconi, G., Ascenzi, P., Bolognesi, M., Guarneri, M. and Menegatti, E. (1987) *Adv. Biosciences*, **65**, 177–180.
29. Ascenzi, P., Aducci, P., Amiconi, G., Bsllio, A., Guaragna, A., Menegatti, E., Schnebli, H.-P. and Bolognesi, M. (1991) *J. Mol. Recognition*, **4**, 113–119.
30. Ascenzi, P., Amiconi, G., Coletta, M., Lupidi, G., Menegatti, E., Onesti, S. and Bolognesi, M. (1992) *J. Mol. Biol.*, **225**, 177–184.
31. Fioretti, E., Angeletti, M., Citro, G., Barra, D. and Ascoli, F. (1987) *J. Biol. Chem.*, **262**, 3586–3589.