ELSEVIER

Biophysical Chemistry 54 (1995) 75–81

Biophysical Chemistry

Inhibition of bovine β-trypsin, human α-thrombin and porcine pancreatic β-kallikrein-B by 4′,6-diamidino-2-phenylindole, 6-amidinoindole and benzamidine: a comparative thermodynamic and X-ray structural study

Elena Casale [a,b], Charles Collyer [b], Paolo Ascenzi [c], Gianni Balliano [d], Paola Milla [d], Franca Viola [d], Mauro Fasano [e], Enea Menegatti [f], Martino Bolognesi [a,g,*]

[a] *Department of Genetics and Microbiology, University of Pavia, Via Abbiategrasso 207, 27100 Pavia, Italy*
[b] *Farmitalia Carlo Erba, Research Centre, Via Giovanni XXIII 23, 20014 Nerviano, Milan, Italy*
[c] *Department of Pharmaceutical Chemistry and Technology, University of Torino, Via Pietro Giuria 9, 10125 Torino, Italy*
[d] *Institute of Applied Pharmaceutical Chemistry, University of Torino, Corso Raffaello 31, 10125 Torino, Italy*
[e] *Department of Inorganic Chemistry, Physical Chemistry and Chemistry of Materials, University of Torino, Via Pietro Giuria 7, 10125 Torino, Italy*
[f] *Department of Pharmaceutical Sciences, University of Ferrara, Via Fossato di Mortara 17 / 19, 44100 Ferrara, Italy*
[g] *Advanced Biotechnology Centre and Department of Physics, University of Genova, Viale Benedetto XV 10, 16132 Genova, Italy*

Received 1 July 1994; accepted 9 September 1994

Abstract

The inhibitory effect of 4′,6-diamidino-2-phenylindole (DAPI) and 6-amidinoindole on the catalytic properties of bovine β-trypsin (trypsin), human α-thrombin (thrombin) and porcine pancreatic β-kallikrein-B (kallikrein) was investigated (between pH 3.0 and 7.0, $I = 0.1$ M; $T = 30.0 \pm 0.5$°C), and analyzed in parallel with that of benzamidine, commonly taken as a molecular inhibitor model of serine proteinases. Next, the X-ray crystal structure of the trypsin:DAPI complex was solved at 1.9 Å resolution ($R = 0.161$). Over the whole pH range explored, values of the association inhibition constant (K_i) for DAPI and 6-amidinoindole binding to trypsin, thrombin and kallikrein are higher than those found for benzamidine association, suggesting a binding mode of DAPI to the enzyme primary specificity pocket-based on the indole moiety of the inhibitor. On lowering the pH from 5.5 to 3.0, the decrease in affinity for DAPI, 6-amidinoindole and benzamidine binding to trypsin, thrombin and kallikrein reflects the acidic pK shift of the Asp189 invariant residue, present at the bottom of the primary specificity subsite of the serine proteinases considered, from 4.5, in the free enzyme, to 3.7, in the proteinase:inhibi-

Abbreviations: Trypsin, bovine β-trypsin; Thrombin, human α-thrombin; Kallikrein, porcine pancreatic β-kallikrein-B; DAPI, 4′,6-diamidino-2-phenylindole; BzArgNHNp, N-α-benzoyl–L-arginine *p*-nitroanilide; PhePipArgNHNp, N-α-D-phenyalanine–L-pipecoline–L-arginine *p*-nitroanilide (S-2238); TosGlyProArgNHNp, N-α-tosyl-glycine–L-proline–L-arginine *p*-nitroanilide (Chromozym-TH)

* Corresponding author.

SSDI 0301-4622(94)00108-1

tor complexes. Inspection of the refined crystal structure of the trypsin:DAPI complex, however, does not allow a unique interpretation of the inhibitor binding mode. The present data were analysed in parallel with those reported for related serine (pro)enzyme/inhibitor systems.

Keywords: Bovine β-trypsin; Human α-thrombin; Porcine pancreatic β-kallikrein-B; 4′,6-Diamidino-2-phenylindole; Thermodynamics of serine proteinase:inhibitor complex; X-ray crystal structure

1. Introduction

Serine proteinases play a central role in several physiological processes, spanning from digestion to key regulatory mechanisms such as peptide hormone release, coagulation and complement activation. They are also recognized as pathogenic factors in many diseases, including tumors, inflammatory processes, pulmonary emphysema, glomerulonephritis, acute pancreatitis and muscular dystrophy [1,2]. The possibility of selectively influencing serine proteinase activities by specific inhibitors appears, therefore, to be of considerable interest in view of their potential therapeutic value as drugs [3].

The development of several synthetic inhibitors of serine proteinases has been based on the discovery of the effectiveness of benzamidine as an anti-trypsin agent by Mares-Guia and Shaw in 1965 [4]. Significant progress in the development of this class of compounds has been brought about by the discovery that the strength and specificity of the inhibitory effect also depends on the remainder (in addition to the benzamidine moiety) of the chemical structure. Thus, the synthetic benzamidine derivatives of N-α-arylsulfonyl-4-amidinophenylalanine show K_i values ranging between 1.0×10^{-9} M and 1.0×10^{-5} M [5]. Of the benzamidine derivatives, 4′,6-diamidino-2-phenylindole (DAPI) seems a peculiar case, given its wide field of applications in biophysical studies. Indeed, DAPI is a fluorescent dye, capable of binding to double stranded DNA with strong enhancement of the fluorescence quantum yield [6]. In particular, DAPI binds in the minor groove of AT-rich sequences, and, to a lesser extent, to GC-rich regions as intercalating compounds [7]. Moreover, interactions of DAPI with bovine serum albumin, negatively charged proteins and phospholipid vesicles or micelles have been reported [8,9]. More specific inhibitory interactions of amidino-substituted aromatic heterocyclic compounds (i.e. DAPI) towards trypsin-like serine proteinases have also been shown [10].

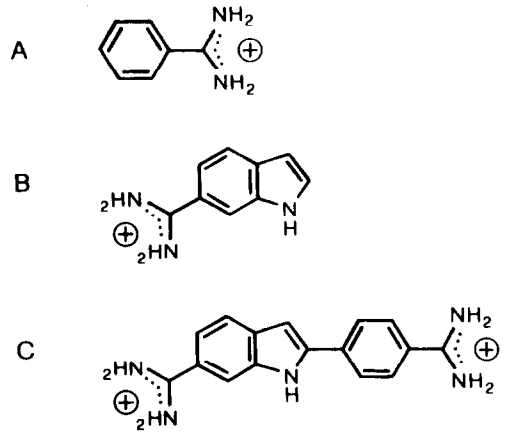

Fig. 1. Chemical structures of benzamidine (A), 6-amidinoindole (B) and DAPI (C).

In order to shed more light on the inhibitory mechanism of benzamidine derivatives, the effect of pH (between pH 3.0 and 7.0; $I = 0.1$ M) on the values of the association inhibition constant (K_i) for the binding of DAPI, 6-amidinoindole and benzamidine (see Fig. 1) to trypsin, thrombin and kallikrein was investigated at 30.0 ± 0.5°C. Next, the X-ray crystal structure of the trypsin:DAPI complex was solved and refined at 1.9 Å resolution allowing the binding behaviour of DAPI, 6-amidinoindole and benzamidine to be related to the stereochemistry of the enzyme:inhibitor contact region(s). The present data have been analysed in parallel with those reported for related serine (pro)enzyme/inhibitor systems [11,12].

2. Materials and methods

Trypsin, treated with diphenylcarbamyl chloride in order to abolish chymotryptic activity, was purified from commercial preparations (from Sigma Chemical Co., St. Louis, MO, USA) [13]. Thrombin

was purified from commercial preparations (from Sigma Chemical Co., St. Louis, MO, USA) [14]. Kallikrein was isolated from commercial preparations (a kind gift of Bayer AG, Wuppertal, Germany) [15]. BzArgNHNp, PhePipArgNHNp, TosGlyProArgNHNp, DAPI and benzamidine were obtained from Sigma Chemical Co. (St. Louis, MO, USA). 6-amidinoindole was synthesized as detailed elsewhere [10]. Deuterated methanol was purchased from Merck AG (Darmstadt, Germany). All chemicals were of analytical grade and used without further purification. The characterization of trypsin, thrombin, kallikrein, BzArgNHNp, PhePipArgNHNp, TosGlyProArgNHNp, DAPI, 6-amidinoindole and benzamidine have been reported elsewhere [10,13–16].

Crystals of the trypsin:DAPI complex were obtained by soaking benzamidine-free trypsin crystals in DAPI saturated solutions containing 2.5 M ammonium sulfate, 1 mg/ml $CaCl_2$, in 0.05 M tris/HCl buffer, pH 8.0 for 15 days, at 4°C. Diffracted intensities were collected on a MarResearch imaging plate system, mounted on a Rigaku RU200 X-ray generator, run at 45 kV, 120 mA. The measured intensities, to a limiting resolution of 1.9 Å, were processed using the MOSFLM data reduction package [17]. A total of 81852 intensities were measured and reduced to 16996 independent reflections (R_{merge} = 0.064, 76.4% completeness in the 10.0–1.9 Å resolution range).

The atomic coordinates of the trypsin:benzamidine complex were recovered from the Brookhaven Protein Data Bank file 1TLD [18] and used as such for phase calculations, once the benzamidine structure and the neighboring sulfate ion were removed from the atomic coordinates. An initial R factor of 0.254 was calculated when the trypsin:DAPI complex structure factors were used. The crystallographic refinement of the complex was performed using the TNT restrained refinement program suite [19] and FRODO [20] for model correction and map inspection. The final crystallographic R-factor after three refinement cycles was 0.161, with nearly ideal values for the model stereochemical parameters (r.m.s. deviations: bond length = 0.019 Å, bond angles = 2.9°).

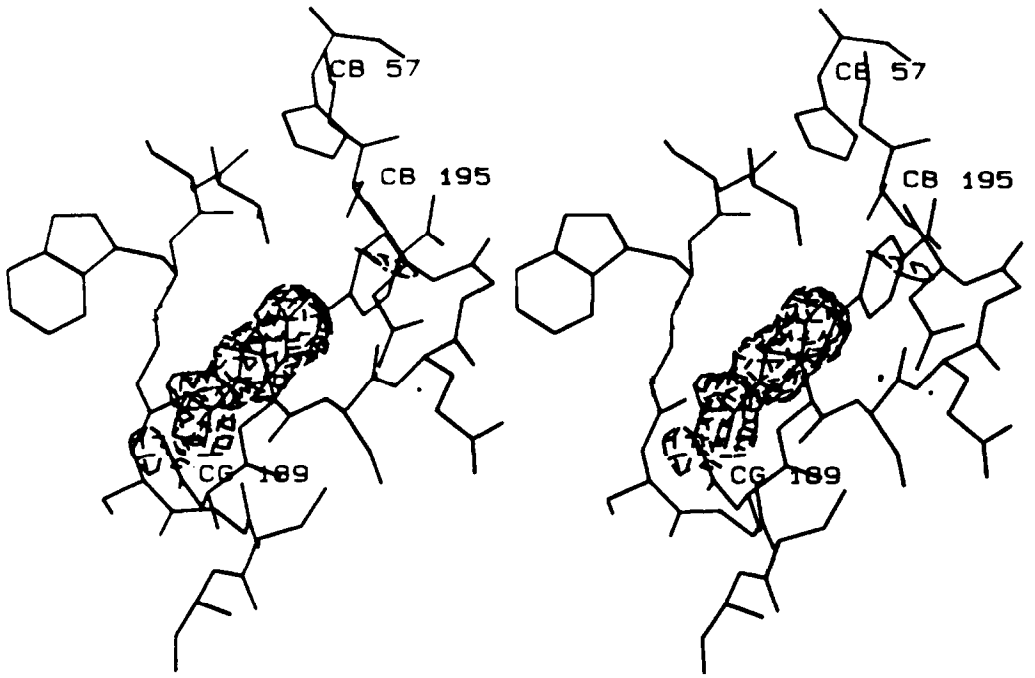

Fig. 2. Stereo view of the trypsin active center around the S_1 subsite, as observed in the crystal structure of the trypsin:DAPI complex. The inhibitor molecule is fitted to its electron density (2Fo-Fc map) in one of the two alternative binding modes (for details see text).

The ^{1}H-NMR spectra of DAPI were collected on a JEOL EX-400 Fourier Transform Nuclear Magnetic Resonance spectrometer, operating at a Proton Larmor Frequency of 399.6 MHz, using deuterated methanol as solvent. The proton resonances of DAPI were assigned as previously reported [21].

Values of the association inhibition constant (K_i) for DAPI, 6-amidinoindole and benzamidine binding to trypsin, thrombin and kallikrein were determined between pH 3.0 and 7.0 (formate buffer, pH 3.0 to 3.5; acetate buffer, pH 3.5 to 6.0; and phosphate buffer, pH 6.0 to 7.0; all at $I = 0.1$ M; sodium salts), and at 30.0 ± 0.5°C, by assay systems using BzArgNHNp as substrate for trypsin and kallikrein, and PhePipArgNHNp and TosGlyProArgNHNp as substrates for thrombin [10,14,16,22,23]. Values of K_i were obtained using the graphical method of Ascenzi et al. [24]; an average error value of $\pm 8\%$ was evaluated for K_i values. The biochemical procedures have been published previously [10,14,16,22, 23].

3. Results and discussion

Inspection of the inhibited enzyme refined crystal structure shows that DAPI binds to the trypsin S_1 specificity subsite (Fig. 2). The detailed analysis of the electron density, however, does not allow a unique interpretation for the inhibitor binding mode. In particular, continuous electron density is present in the inner part of the S_1 pocket, close to Asp189 carboxylate, compatible with the presence of a guanidino group salt-linked to trypsin Asp189 residue. Moreover, two hydrogen bonds, 2.75 Å and 2.88 Å respectively, are observed between the guanidino nitrogen atoms and Asp189 carboxylate.

On the other hand, the inhibitor electron density gets weaker moving towards the solvent exposed protein surface, not accounting for the whole DAPI molecule (Fig. 2). These observations can account for the binding of DAPI to trypsin S_1 subsite either through the 6-amidinoindole or through the benzamidine moiety.

In both cases rotation of the solvent exposed inhibitor substructure is probably the main cause of electron density perturbation outside the S_1 pocket. In this respect, the ^{1}H-NMR spectra of DAPI in methanolic solution in the -30 to $+50$°C temperature range show that the degenerations of 3′-5′- and 2′-6′-protons are not resolved. On decreasing the temperature, only a generalized broadening of the signals is observed, due to the increased viscosity of the solution. The small chemical shift variation indicates only the presence of hydrogen bonds involving the amidino groups close to the observed aromatic protons, and does not allow the establishment of any conformation exchange process between the two pairs of protons on the benzamidine ring, which would be anisochronous if the conformation of the molecule were rigid on the NMR timescale. Thus, the ^{1}H-NMR spectra show a fast motion of the benzamidine ring, relative to the 6-amidinoindole moiety of DAPI, in the temperature range considered.

For all the serine proteinase/inhibitor systems considered, the inhibition patterns were strictly competitive, and the complex formation conformed to simple equilibria, as indicated by the unitary value of the Hill coefficient ($n = 1.00 \pm 0.02$). Under all the experimental conditions, values of K_i for DAPI, 6-amidinoindole and benzamidine binding to trypsin, thrombin and kallikrein were independent of the serine proteinase, substrate and inhibitor concentration, and compared well with those reported in the literature [10,22,25,26]. According to Menegatti et al. [22], no specific ion effects were found using different buffers with overlapping pH values.

Data shown in Fig. 3 allow the following considerations: (i) DAPI and 6-amidinoindole bind to the serine proteinases considered with the same affinity. This finding would suggest that the 6-amidinoindole moiety of DAPI sits in the S_1 subsite of the proteinase active center. (ii) Values of K_i for DAPI and 6-amidinoindole binding to trypsin, kallikrein and thrombin are higher than those observed for the serine proteinase:benzamidine adduct formation. Such a behaviour could be related to more extended van der Waals contacts achieved in the enzyme S_1 pocket upon burying of the indole system of DAPI, with respect to benzamidine. (iii) The affinity of benzamidine for trypsin is higher than that observed for inhibitor binding to thrombin and kallikrein. Next, values of K_i for DAPI and 6-amidinoindole binding to trypsin and thrombin are higher than those observed for kallikrein. Accordingly, the binding of benzamidine (and possibly DAPI and 6-amidinoin-

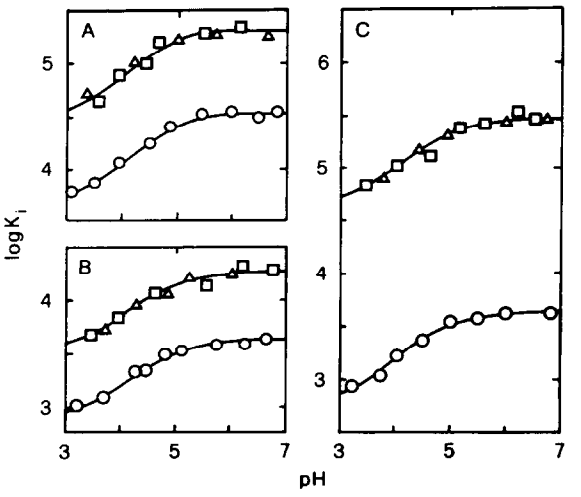

Fig. 3. pH dependence of K_i (M^{-1}) for DAPI (squares), 6-amidinoindole (triangles) and benzamidine (circles) binding to trypsin (panel A), kallikrein (panel B) and thrombin (panel C), at 30.0 ± 0.5°C. The unbroken lines, generated according to Eq. (1) with the following sets of parameters: trypsin/benzamidine — $C = 4.52$; $pK_{UNL} = 4.50$ and $pK_{LIG} = 3.80$; trypsin/DAPI and /6-amidinoindole — $C = 5.32$, $pK_{UNL} = 4.50$ and $pK_{LIG} = 3.70$; kallikrein/benzamidine — $C = 3.60$, $pK_{UNL} = 4.45$ and $pK_{LIG} = 3.75$; kallikrein/DAPI and /6-amidinoindole — $C = 4.27$, $pK_{UNL} = 4.50$ and $pK_{LIG} = 3.80$; thrombin/benzamidine — $C = 3.62$, $pK_{UNL} = 4.50$ and $pK_{LIG} = 3.70$; and thrombin/DAPI and /6-amidinoindole — $C = 5.45$, $pK_{UNL} = 4.50$ and $pK_{LIG} = 3.70$, were obtained with an iterative non-linear least-squares curve fitting procedure, which also allowed to ascribe an average error value of $\pm 12\%$ to values of 10^C, K_{UNL} and K_{LIG}, as the standard deviation. For details, see text.

dole) to the kallikrein S_1 subsite is hampered by structural perturbations of the enzyme specificity pocket [27].

On lowering the pH from 5.5 to 3.0, the decrease in affinity (i.e., in K_i values) for DAPI, 6-amidinoindole and benzamidine binding to trypsin, thrombin and kallikrein reflects, according to linkage relations [28], the acidic pK shift of a single ionizing group on the serine proteinase:inhibitor complex formation. This model leads to the following expression (Eq. 1) [22]:

$$\log K_i = C - \log \frac{[H^+] + K_{UNL}}{[H^+] + K_{LIG}} - \log \frac{K_{LIG}}{K_{UNL}} \qquad (1)$$

where C is a constant corresponding to the neutral asymptote of log K_i, and pK_{UNL} and pK_{LIG} are pK values of the proton dissociation equilibrium constants for the inhibitor-free (K_{UNL}) and the inhibitor-bound (K_{LIG}) serine proteinase, respectively. Eq. (1) has been used to generate the unbroken lines shown in Fig. 3; the agreement with the experimental data is fully satisfactory, giving us confidence on the correct assumption(s) underlying Eq. (1) (see Fig. 3).

As already reported for the binding of substrates and inhibitors, showing a cationic residue at the P_1 position, to trypsin-like serine proteinases (see Refs. [10,29–31]), DAPI, 6-amidinoindole and benzamidine preferably bind the unprotonated species of trypsin, thrombin and kallikrein.

Values of pK_{UNL} and pK_{LIG} fitting DAPI, 6-amidinoindole and benzamidine binding to trypsin, thrombin and kallikrein are closely similar for the enzymes investigated (see Fig. 3), and agree very well with those of amino acid residue(s) modulating, between pH 3.0 and 5.5, catalytic, inhibitor binding and spectral properties of serine (pro)enzymes acting on cationic substrates (see Refs. [29–31]).

Inspection of the X-ray three dimensional structures [27,32–34] and computer-generated molecular models (see also Refs. [22,26,30]) of the enzyme:inhibitor complexes suggests that the ionizable group affecting DAPI, 6-amidinoindole and benzamidine binding to the serine proteinases considered can be identified with the invariant Asp189 residue, present at the S_1 subsite of trypsin, thrombin and kallikrein. In fact, it is known that this residue interacts with the positively charged amidino group of benzamidine in the S_1 subsite of trypsin, thrombin and kallikrein [27,32,34]. As shown by the present data (see Figs. 2 and 3), the same structural and functional considerations can be extended to the association of 6-amidinoindole and of the 6-amidino group of DAPI to the serine proteinases considered.

Previous investigations on bis-, tris- and tetra-benzamidine compounds (1,3-bis(*p*-amidinophenoxy)-propane and derivatives) have shown that the additional amidino group(s) do not provide means of additional inhibitor:enzyme interaction in the case of trypsin. On the other hand, the same compounds show higher affinities for thrombin and kallikrein, as compared to benzamidine, the increase in enzyme inhibitory effect being related to the presence of a second benzamidine substituent in the molecule [25,26]. Absence of additional interactions (with re-

spect to benzamidine or 6-amidinoindole) in DAPI:enzyme complexes is related to the difference in conformational freedom observed for DAPI and for poly-benzamidine substituted compounds. Thus, adaptation to the protein surface allows 1,3-bis(*p*-amidinophenoxy)-propane to reach both Asp189 and Glu146 (or Glu192), on thrombin surface [26]. On the contrary, such an interaction is precluded to DAPI, which, in this context, is a planar molecule not capable of the required conformational readjustments.

As a whole, if there is to be inhibitor binding discrimination among homologous macromolecular species (such as trypsin, thrombin and kallikrein), simple chemical modification(s) may confer the desired specificity. The present results suggest that a fine degree of recognition in the association of synthetic inhibitors to serine proteinases may be obtained through modulation of the interaction(s) occurring at the enzyme S_1 specificity pocket. From this viewpoint, DAPI, 6-amidinoindole and benzamidine association to serine proteinases appears to be a paradigmatic model for the study of interactions selectively occurring at a given (pro)enzyme:inhibitor recognition subsite.

Acknowledgements

The authors thank Prof. Silvio Aime for helpful discussions. The present study was partially supported by grants from the Ministry of University, Scientific Research and Technology of Italy (MURST), from the National Research Council of Italy (CNR, Target Oriented Projects Biotecnologie e Biostrumentazione and Chimica Fine II), and from Centro Interuniversitario Macromolecole Informazionali (Pavia, Italy).

References

[1] H. Hörn and A. Heidland (Editors), Proteases: Potential Role in Health and Diseases, Plenum Press, New York, 1992.
[2] D.D. Cunningham and G.L. Long (Editors), Proteinases in Biological Control and Biotechnology, Alan R. Liss Inc., New York, 1987.
[3] A.J. Barrett and G. Salvesen (Editors), Proteinase Inhibitors, Elsevier, Amsterdam, 1986.
[4] M. Mares-Guia and E. Shaw, J. Biol. Chem., 240 (1965) 1579.
[5] J. Strüzebecher, F. Markwardt, B. Voigt, G. Wagner and P. Walsmann, Thromb. Res., 29 (1983) 635.
[6] J. Kapuscinski and B. Skocrylas, Nucl. Acids Res., 5 (1978) 3775.
[7] W.D. Wilson, F.A. Tanious, H.J. Barton, R.L. Jones, K. Fox, R.L. Wydra and L. Strekowski, Biochemistry, 29 (1990) 8452.
[8] A. Mazzini, P. Cavatorta, M. Ioni, R. Favilla and G. Sartor, Biophys. Chem., 42 (1992) 101.
[9] R. Favilla, R. Stecconi, P. Cavatorta, G. Sartor and A. Mazzini, Biophys. Chem., 46 (1993) 217.
[10] J.D. Geratz, F.M. Stevens, K.L. Polakoski, R.F. Parrish and R.R. Tidwell, Arch. Biochem. Biophys., 197 (1979) 551.
[11] W. Bode and R. Huber, Curr. Opinion Struct. Biol., 1 (1991) 45.
[12] W. Bode and R. Huber, Eur. J. Biochem., 204 (1992) 433.
[13] J.A. Luthy, M. Praissman, W.R. Finkenstadt and M. Laskowski Jr., J. Biol. Chem., 248 (1973) 1760.
[14] J. Elion, J.-P. Boissel, B. Le Bonniec, A. Bezeaud, M. Jondrot-Perrus, M.-J. Rabiet and M.-C. Guillin, Ann. NY Acad. Sci., 485 (1986) 16.
[15] F. Fiedler, E. Fink, H. Tschesche and H. Fritz, Methods Enzymol., 80 (1981) 493.
[16] R. Lottenberg, U. Christensen, C.M. Jackson and P.L. Coleman, Methods Enzymol., 80 (1981) 341.
[17] A.G.W. Leslie, P. Brick and A.J. Wonnacott, CCP4 News, 18 (1986) 33.
[18] F.C. Bernstein, T.F. Koetzle, G.J.B. Williams, E.F. Meyer Jr, M.D. Brice, J.R. Rodgers, O. Kennard, T. Shimanouchi and M. Tasumi, J. Mol. Biol., 112 (1977) 535.
[19] D.E. Tronrud, L.F. Ten Eyck and B.W. Matthews, Acta Crystallogr. Sect. A, 43 (1987) 489.
[20] T.A. Jones, J. Appl. Crystallogr., 11 (1978) 268.
[21] W.D. Wilson, F.A. Tanious, H.J. Barton, L. Strekowski, D.W. Boykin and R.L. Jones, J. Am. Chem. Soc., 11 (1989) 5008.
[22] E. Menegatti, R. Ferroni, S. Scalia, M. Guarneri, M. Bolognesi, P. Ascenzi and G. Amiconi, J. Enzyme Inhibition, 2 (1987) 23.
[23] S.R. Stone, P.J. Braun and J. Hofsteenge, Biochemistry, 26 (1987) 4617.
[24] P. Ascenzi, M.G. Ascenzi and G. Amiconi, Biochem. Educ., 15 (1987) 134.
[25] E. Menegatti, M. Guarneri, R. Ferroni, M. Bolognesi, P. Ascenzi and E. Antonini, FEBS Lett., 141 (1982) 33.
[26] P. Ascenzi, R. Fruttero, G. Amiconi, L. Pugliese, M. Bolognesi, M. Coletta, S. Onesti, M. Guarneri and E. Menegatti, J. Enzyme Inhibition, 6 (1992) 131.
[27] W. Bode, Z. Chen, K. Bartels, C. Kutzback, G. Schmidt-Kastner and H. Bartunik, J. Mol. Biol., 164 (1983) 237.
[28] J. Wyman, Advan. Protein Chem., 19 (1964) 223.
[29] G. Amiconi, P. Ascenzi, M. Bolognesi, E. Menegatti and M. Guarneri, in I. Chaiken, E. Chiancone, A. Fontana and P.

Neri (Editors), Macromolecular Biorecognition, Humana Press, Clifton, 1988, p. 117.
[30] M. Bolognesi, P. Ascenzi, G. Amiconi, E. Menegatti and M. Guarneri, in I. Chaiken, E. Chiancone, A. Fontana and P. Neri (Editors), Macromolecular Biorecognition, Humana Press, Clifton, 1988, p. 81.
[31] E. Menegatti, M. Guarneri, M. Bolognesi, P. Ascenzi and G. Amiconi, in I. Chaiken, E. Chiancone, A. Fontana and P. Neri (Editors), Macromolecular Biorecognition, Humana Press, Clifton, 1988, p. 101.
[32] W. Bode and P. Schwager, J. Mol. Biol., 98 (1975) 693.
[33] W. Bode, I. Mayr, U. Baumann, R. Huber, S.R. Stone and J. Hofsteenge, EMBO J., 8 (1989) 3467.
[34] D.W. Banner and P. Hadvary, J. Biol. Chem., 226 (1991) 20085.