Synergistic binding of inhibitors to the protease from HIV type 1

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Inhibition of the protease in HIV is a potentially useful approach for the treatment of AIDS. In the course of evaluating inhibitors of the HIV-1 protease, we observed a strong synergism between certain inhibitors that might be expected to bind to different sites in this enzyme. The binding affinity of carbobenzyloxyisoleucinyphenylalaninol, for example, is increased 125-fold in the presence of carbobenzyloxylglutaminyloamylamide. These synergistic effects between inhibitors have specific structural requirements

that correlate well with the known substrate preference of the enzyme. The molecular basis for this phenomenon remains to be elucidated but it could involve substrate-induced conformational change as part of the reaction mechanism. Similar effects have been reported previously for several zinc proteases. Thus this work extends the observation to a different class of enzymes and suggests that the phenomenon might be widespread.

INTRODUCTION

Efforts to develop a treatment for AIDS have drawn attention to enzymes required for the replication of HIV type 1 (HIV-1) [1]. The vital role of the HIV-1 protease in the post-translational processing of precursors into viral proteins [2,3] has established the enzyme as an attractive target for the design of drugs against AIDS. The extensive knowledge previously obtained about other aspartate proteases coupled with the recent intensive research on HIV-1 protease have resulted in the development of a number of potent inhibitors [4–7].

For the design of enzyme inhibitors, an obvious goal is to maximize affinity by using all the potential interactions available. In the HIV-1 protease, these interactions are likely to be numerous and intricate because of the highly extended nature of its active site. Thus, judging from the minimum length required for peptides to act as substrates, the enzyme seems to be capable of recognizing at least three amino acid residues on either side of the scissile bond [8]. Furthermore the substrate specificity of the enzyme shows a complexity that cannot be accounted for solely in terms of binding preferences at individual subsites [9]. X-ray crystallographic studies of enzyme–inhibitor complexes also suggest that substrate recognition is a complicated process involving many residues [10–12]. Presumably, there are subtle interactions between different regions of the active site that influence the catalytic process.

To investigate the possible existence of such interactions between subsites we have examined whether two inhibitors binding to different locations could exert effects on each other. In the past few years our laboratory has investigated the phenomenon of synergism in the binding of inhibitors to several zinc proteases [13–15] including the clinically important angiotensin-converting enzyme [16]. Although inhibitor synergism has not been reported in aspartate proteases, theoretical considerations suggest that it should be observable in a variety of enzymes [15]. We therefore set out to investigate whether similar effects could be found in the aspartate protease from HIV-1 in view of the widespread interest in this enzyme.

MATERIALS AND METHODS

All chemicals were of the highest purity commercially available. The affinity-purified recombinant HIV-1 protease was purchased

from Bachem (Philadelphia, PA, U.S.A.). The HIV-1 protease substrate, AcSQNYPVV-amide, was custom synthesized by Pharmacia (Toronto, Ontario, Canada). All inhibitors were synthesized by using established coupling methods with dicyclohexycarbodi-imide and hydroxybenzotriazole for peptide synthesis [17,18]. The purity of compounds was ascertained by TLC and HPLC. The compounds were characterized by ¹H NMR, mass spectroscopy and melting point determinations. ¹H NMR spectra were recorded on either a Bruker 200 or 500 MHz spectrometer. A Fisher–Johns melting point apparatus was used to determine melting points.

Enzyme assay

The activity of HIV-1 protease was assayed by using a modified version of the methods described in [8,19]. Enzyme activity was determined at 37 °C in a 50 µl reaction volume containing 50 mM Mes, pH 6, 1 mM EDTA, 1 mM dithiothreitol, 10% (v/v) glycerol, 5% (v/v) DMSO, 200 mM NaCl, 0.5 mM substrate and 0.8 ng/µl HIV-1 protease. The products from a 20 min reaction were quenched with 75 µl of 12% acetic acid and separated by reverse-phase HPLC on a C-18 Separon column (Fisher) with a linear gradient of 5–30% (v/v) acetonitrile at a flow rate of 1 ml/min. Reaction rates were computed from integrated peaks detected at 230 nm and corresponded to the AcSQNY cleavage product. The observed values were linear with time for at least 80 min and proportional to enzyme concentration up to four times the level described above. The *K_m* for the substrate AcSQNYPVV-amide (6.5 mM) under the above assay conditions was comparable to that (7.5 mM) measured previously [8] in the absence of DMSO. HPLC analysis was performed on either a Beckman 421 or a Beckman System Gold unit.

Following the method of Yonetani and Theorell [20], we analysed the concurrent effect of two inhibitors by varying independently the concentration of each inhibitor while keeping the substrate and enzyme concentrations constant. The resulting data can be analysed graphically (an example is shown in Figure 1) to yield an interaction constant (α) that expresses the influence of one inhibitor on the affinity of the enzyme for the other. As originally defined [20], this constant is the ratio between the

Abbreviation used: Cbz, carbobenzyloxy.

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Figure 1 Analysis of the inhibition data by the method of Yonetani and Theorell [20]

The reciprocal of enzyme activity is plotted against the concentration of one inhibitor (Cbz-Gln-isoamylamide in this case). The various lines represent different concentrations of the second inhibitor (Cbz-Ile-phenylalaninol): ●, 7.5 μM ; ○, 15 μM ; ▲, 22.5 μM ; △, 30 μM . Note that the concentration ranges selected for both inhibitors were much lower than the K_i values (8.9 and 0.29 mM) because of the unusually low value of the interaction constant, α , in this case (0.008; see Table 3); α can be calculated from the intersection point, which corresponds to an inhibitor concentration equal to $-\alpha K_i$. For assay conditions, see the Materials and methods section.

dissociation constant for the ternary complex involving the enzyme and the two inhibitors, and the dissociation constant for the binary complex:

It follows that synergism is observed when α is significantly smaller than 1 because this corresponds to a decrease in the apparent dissociation constant for the ternary complex. At the other extreme, α approaches infinity if the two inhibitors are mutually exclusive. In contrast, if the binding occurs independently at separate sites then α should have a value of unity. Inhibition constants (K_i) were normally determined by means of Dixon plots [21] with four different concentrations of the inhibitor while varying substrate concentration from 0.5 to 2 mM. In situations where the K_i of an inhibitor was fairly high compared with its solubility, the K_i values were indirectly estimated from the Yonetani–Theorell plots as previously described [16].

RESULTS

Design of synergistic probes

To detect possible synergism in HIV-1 protease it was necessary to find two probes that might be expected to bind in different regions of the active site. Our approach was to examine previously known inhibitors and determine how the structure in each case might be divided into two suitable fragments. A promising candidate was the symmetrical and highly potent inhibitor,

Figure 2 Structures of inhibitors and expected binding modes

(a) The symmetrical inhibitor A74704 [22]. (b) The hydroxy fragment (I, Cbz-Val-phenylalaninol) and the alkylamide fragment (II, Cbz-Val-phenethylamide) aligned in the same way as the intact inhibitor above. For comparison, the corresponding positions of the scissile bond and various residues of the substrate are shown below the structures. The direction of the backbone is indicated as $N \rightarrow C$. (c) An alternative binding mode for the alkylamide fragment.

A74704 ($K_i = 4.5$ nM) reported by a group at the Abbott Laboratories [22]. As shown in Figure 2, this inhibitor contains a hydroxy group that is expected to mimic the scissile peptide bond in a tetrahedral intermediate. This molecule can be divided conveniently into two almost equal fragments (Figure 2b), each possessing enough structural characteristics to bind specifically to distinct regions of the active site. Experimentally, these two probes have the advantage of being amenable to simple synthesis from readily available materials. Variation of the substituents at different parts of each probe could easily be accomplished. For convenience, the two types of probe will be referred to subsequently as the hydroxy and alkylamide fragments (structures (i) and (ii) in Figure 2b) respectively.

Binding mode of the fragments

When the above two fragments were evaluated for synergism, a value of 0.1 was obtained for α , indicating that the affinity of the enzyme for either inhibitor is increased 10-fold ($1/\alpha$) by the presence of the other. Because the hydroxy group would most probably occupy the normal position of the scissile bond, the fragment containing this group is expected to bind in a specific orientation with the phenylalanine side chain representing the P_1 residue (using the notation of Schechter and Berger [23] for designating protease substrates). The peptide backbone of this inhibitor should then have the same alignment as the N-terminal portion of the substrate (Figure 2b). The binding mode of the alkylamide fragment, on the other hand, is subject to some uncertainty. If we assume that the two fragments are bound in the same manner as the original intact inhibitor (as determined by X-ray crystallography) [24], then the C_2 symmetry of the

Table 1 Effects of varying the alkylamide fragment on its synergism with Cbz-Val-phenylalaninol

For consistency in comparison, all values of the interaction constant, α , were calculated by using the K_i value (0.25 ± 0.01 mM) for Cbz-Val-phenylalaninol.

Alkylamide fragment	K_i (mM)	α
Cbz-Val-phenethylamide	0.07 ± 0.03	0.1 ± 0.1
Cbz-Val-isoamylamide	0.20 ± 0.05	0.4 ± 0.2
Cbz-Val-benzylamide	0.05 ± 0.02	0.9 ± 0.2
Cbz-Gln-phenethylamide	2.3 ± 0.60	0.13 ± 0.05
Cbz-Gln-isoamylamide	8.9 ± 1.2	0.014 ± 0.005

parent molecule dictates that the alkylamide fragment be oriented in the opposite direction to the substrate (Figure 2b). This would place the phenethyl group and the carbobenzyloxy (Cbz) group in the P_1' and P_3' positions, respectively. However, it is possible that binding induces an asymmetry in the enzyme such that both fragments are oriented with their peptide backbone in the same direction. In that case, the above assignment of P_1' and P_3' sites would be exactly reversed (Figure 2c). Because the Cbz group is virtually identical with the phenethyl group in terms of size and chemical character, the binding preferences of subsites have little influence on the orientation of the fragment in this instance. In considering the binding mode of the above probes, it must be remembered that covalent bonds are always shorter than the corresponding van der Waals radii. Therefore the fragments could never be bound in precisely the same location as the original intact molecule.

Structural requirements for synergism

Having detected synergism in the above pair of fragments, we proceeded to vary the structure of the alkylamide fragments to analyse the effects on synergism. The range of possible structural variations was, however, often limited by the low solubility of the inhibitors despite the inclusion of 5% DMSO in the assay. This problem was particularly severe for the alkylamide fragment, which was generally less soluble (as little as one-tenth). Thus useful data could be obtained only if the inhibitors had a relatively high affinity for the enzyme or showed pronounced synergism. As shown in Table 1, the alkylamide substituent has a significant role in the phenomenon. When the phenethyl group was replaced by an isoamyl moiety, a substantial reduction in synergism occurred. A change to the benzyl group further resulted in the disappearance of almost all synergistic effects. We have also synthesized some compounds containing a smaller substituent in this position (e.g. Cbz-Val-isobutylamide and Cbz-Asn-methylamide), but they proved to be poor inhibitors of the enzyme (results not shown). The above difference between the phenethyl and isoamyl groups in producing synergism was found to be dependent on the nature of the neighbouring substituent (the putative P_2' site). Thus when the valine residue in this position was replaced by glutamine (Table 1), the effects were reversed, with the isoamylamide derivative showing a much higher synergism than the corresponding phenethylamide inhibitor. This finding is consistent with the complexity observed in the substrate specificity of the enzyme and suggests a subtle relationship between subsites. Alternatively, a change in the binding mode (as explained above) could be responsible for the difference. Overall there is no appreciable correlation between binding affinity and the degree of synergism. For example, in the phenethylamide series, the K_i values of the Val and Gln deriv-

Table 2 Effects of the putative P_2' residue in the alkylamide fragment on its synergism with Cbz-Val-phenylalaninol

Experimental conditions are the same as for Table 1.

Alkylamide fragment	K_i (mM)	α
Cbz-Asn-isoamylamide	2.3 ± 0.8	0.05 ± 0.01
Cbz-Gly-isoamylamide	4.2 ± 1	1.2 ± 0.3
Cbz-Leu-isoamylamide	0.15 ± 0.06	0.4 ± 0.2
Cbz-Ile-isoamylamide	0.05 ± 0.02	0.13 ± 0.05
Succinamyl-isoamylamide	4.1 ± 0.6	0.21 ± 0.03

Table 3 Effects of varying the P_2 residue in the hydroxy fragment on its synergism with Cbz-Gln-isoamylamide

For consistency in comparison, all values of interaction constants were calculated by using the K_i value for Cbz-Gln-isoamylamide (8.9 mM). The enhanced affinity of Cbz-Ile-phenylalaninol in the presence of Cbz-Gln-isoamylamide is given by αK_i , which equals $2.3 \mu\text{M}$. Other experimental conditions are described in the Materials and methods section.

Hydroxy fragment	K_i (mM)	Interaction constant (α)
Cbz-Val-phenylalaninol	0.25 ± 0.01	0.014 ± 0.005
Cbz-Ile-phenylalaninol	0.29 ± 0.08	0.008 ± 0.001
Cbz-Leu-phenylalaninol	0.4 ± 0.2	0.07 ± 0.02
Cbz-Gln-phenylalaninol	0.6 ± 0.6	0.09 ± 0.02
Cbz-Asn-phenylalaninol	0.6 ± 0.1	0.13 ± 0.03

atives differ by more than 30-fold but their α values (for the interaction with the same hydroxy fragment) were not significantly different.

The very high synergism obtained above with Cbz-Gln-isoamylamide correlates well with modelling studies [25], which showed a preference for Glu or Gln at the P_2' position in substrates and prompted us to examine similar alkylamide fragments with other substituents (Table 2). The Asn derivative was also quite effective in producing synergism, although less so than the corresponding fragment containing Gln. However, no synergism was observed with glycine in this position, indicating the importance of the side chain. The presence of Leu and Ile side chains resulted in moderately synergistic effects in the same range as the Val derivative (Table 1). Surprisingly, the Cbz group could be omitted in a truncated fragment without abolishing synergism completely (in this case the succinamyl group represents the Asn side chain in the P_2' position).

To study further the relationship between structure and synergism we varied the P_2 residue in the hydroxy fragment. The other inhibitor in these experiments was Cbz-Gln-isoamylamide because this alkylamide fragment showed the highest potential for synergism. An even greater degree of synergism was indeed obtained by replacing the Val substituent with Ile in the hydroxy fragment (Table 3). The interaction constant in this case (0.008) indicates that the binding affinity of each fragment is enhanced 125-fold in the presence of the other. This result approaches the highest level of synergism observed in similar studies [16]. The structural dependence for synergism was very specific because the corresponding Leu derivative was much less effective in this respect. The much stronger synergism shown by the Ile and Val derivatives can be correlated with the apparent preference of the enzyme for β -branched amino acids at the P_2 position in substrates [26]. It is interesting that the presence of Gln in the P_2 position also produced only a moderate degree of synergism.

This finding suggests that maximal effects require a structural asymmetry in the two fragments. Thus the combination of Ile and Gln, respectively, in the putative P_2 and P_2' positions of the two fragments showed an 11-fold greater synergism than the combination with Gln in both positions. Similarly, as reported above (Figure 2 and Table 1), the perfectly symmetrical combination represented by Cbz-Val-phenylalaninol and Cbz-Val-phenethylamide was also only moderately synergistic.

DISCUSSION

Molecular basis of the synergistic effects

The data presented in this paper indicate that the affinity of HIV-1 protease for an active-site probe can in some cases be greatly enhanced by the presence of a second probe binding to another region. Although we do not have any structural information about the interactions of these probes with the enzyme, it is of some interest to consider the possible molecular basis of the above effects. One obvious possibility is that the observed synergism stems from direct interaction between the bound fragments. Because the fragments are fairly hydrophobic in nature, this explanation might superficially seem attractive. Such a direct interaction could result from van der Waals forces between the bound fragments or from the displacement of water from the active site (hydrophobic effect). However, general effects of this nature would not be expected to show the highly specific structural requirements for synergism actually observed. In particular they would not be closely correlated with the substrate specificity of the enzyme. Instead these characteristics of the phenomenon strongly suggest an alternative type of explanation, namely that the effects are indirect and protein-mediated. Only the participation of the precisely constructed binding site of a protein could account for the specific structural requirements for synergism reported here. It follows that the binding affinity of the protein must somehow be enhanced when both fragments are present. Because a single conformation of the protein cannot have two different affinities towards the same ligands, this explanation implies a conformational change as the basis for synergism. It is not necessary for the associated conformational change to be large because the free-energy difference (ΔG) involved in a 125-fold increase in affinity is only -3 kcal/mol. The displacement of a side chain by a small distance would be sufficient to generate the observed effects. Movement of this nature could be rather localized so that it might be difficult to detect by physical methods.

Significance of the phenomenon

In terms of its biological significance, the most relevant observation is that the effects are closely correlated with the substrate specificity of the enzyme. This suggests that the conformational change postulated above is part of the substrate recognition or catalytic process. Such a mechanism could indeed be responsible for the highly complex specificity of HIV-1 protease [27]. However, substrate-induced conformational change is a widely observed phenomenon (see the review by Citri [28]) that might have a more general explanation. We have previously proposed on the basis of similar observations in zinc proteases that the phenomenon could represent a widespread mechanism for enhancing reaction rates [15]. The proposed mechanism deals with the problem of using ionizable groups of enzymes for catalysis (e.g. as proton donor or acceptor) in an aqueous medium. By having a suitable pK , such a group can be kept in the required form (protonated or deprotonated as the case may be) as long as water is present. Once the substrate is

bound and water is excluded, the accompanying conformational change can shift the pK of this group to increase its reactivity. This mechanism has been termed the xerophilic shift and is postulated to operate in a wide variety of enzymes [15]. Thus an important aspect of the present study is to extend the observation from zinc proteases to aspartate proteases. Our recent work indicates that pig pepsin also exhibits synergism in its binding of inhibitors, although the structural requirements for these effects are different from those discussed above (E. Asante-Appiah and W. W.-C. Chan, unpublished work). These results therefore support the hypothesis that the above mechanism could be a fairly general feature of enzyme action.

Relevance to drug design

For an enzyme of significant pharmacological interest, the crucial question is whether the synergistic effects reported above could have therapeutic relevance. In general it is unlikely that the administration of two inhibitor fragments as drugs will be preferable to that of the parent compound. Thus, even in the most favourable case shown here, the enhanced affinity of the fragment ($\alpha K_i = 2.3 \mu M$; see Table 3) is still several orders of magnitude weaker than that of the original intact inhibitor ($K_i = 4.5$ nM) [24]. This situation is normally expected to prevail because of the entropic advantage in the interaction of the enzyme with a single molecule [29]. However, the observed effects are still relevant to the optimization of inhibitors because they indicate that the binding energies of the subsites are not additive. Thus in any attempt to increase the affinity of a full-length inhibitor (such as A74704 in Figure 2a), it would be advisable to take into account the mutual interaction of these subsites and not to consider the modification of any individual structural locus in isolation.

Another potential use of the above synergistic effects is to enhance the inactivation of the target enzyme by an irreversible reagent. In the classical approach, a reactive functional group (e.g. the chloromethylketone moiety in serine protease inactivators) is incorporated into a substrate analogue to provide an active-site directed reagent or affinity label. This group must be attached in a position that corresponds approximately to the scissile bond of the substrate. For an endopeptidase such as the HIV-1 protease, the reactive group should therefore be located in the middle of the full-length substrate analogue (e.g. at or near the hydroxy group of the inhibitor A74704). However, covalent modification of active-site residues is known to occur much more readily with functional groups at one end of the molecule [30] presumably because this position allows optimal orientation and better access for chemical attack. In the HIV-1 protease this location of the functional group can be achieved only by using half of the binding site. In other words, the reagents used would be structural analogues of the fragments studied in this work rather than analogues of the full-length substrate. The strategy proposed here is to add a second fragment as a synergistic effector in the expectation that this would increase the inactivation rate substantially. If the increase were of the same order as the synergistic effects observed here (up to 125-fold), it would represent a significant improvement in potency and selectivity. Although this approach has yet to be successfully demonstrated, there are reasonable indications that it should be feasible. As described earlier, a close correlation exists between the structural requirements for inhibitor-binding synergism and catalytic specificity. This correlation suggests that the observed effects are related to the reactivity of the catalytic residues. Thus inactivation should be strongly influenced by the

binding of such a synergistic effector. It is hoped that the present report would stimulate research in these directions.

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REFERENCES

- Mitsuya, H., Yarchoan, R. and Broder, S. (1990) *Science* **249**, 1533–1543
- Kohl, N. E., Emini, E. A., Schleif, W. A., Davis, L. J., Heimback, J. C., Dixon, R. A. F., Scolnick, E. M. and Sigal, I. S. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 4686–4690
- Seelmeier, S., Schmidt, H., Turk, V. and von der Helm, K. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 6612–6616
- Dreyer, G. B., Metcalf, B. W., Tomaszek, T. A., Jr., Carr, T. J., Chandler, A. C., III, Hyland, L., Fakhoury, S. A., Magaard, V. W., Moore, M. L., Strickler, J. E., Debouck, C. and Meek, T. D. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 9752–9756
- McQuade, T. J., Tomasselli, A. G., Liu, L., Karacostas, V., Moss, B., Sawyer, T. K., Heinrikson, R. L. and Tarpley, W. G. (1990) *Science* **247**, 454–456
- Roberts, N. A., Martin, J. A., Kinchington, D., Broadhurst, A. V., Craig, J. C., Dunan, I. B., Galpin, S. A., Handa, B. K., Kay, J., Kröhn, A., Lambert, R. W., Merrett, J. H., Mills, J. S., Parkes, K. E. B., Redshaw, S., Ritchie, A. J., Taylor, D. L., Thomas, G. J. and Machin, P. J. (1990) *Science* **248**, 358–361
- Rich, D. H., Prasad, J. V. N. V., Sun, C.-Q., Green, J., Mueller, R., Houseman, K., MacKenzie, D. and Malkovsky, M. (1992) *J. Med. Chem.* **35**, 3803–3812
- Moore, M. L., Bryan, W. M., Fakhoury, S. A., Magaard, V. W., Huffman, W. F., Dayton, B. D., Meek, T. D., Hyland, L., Dreyer, G. B., Metcalf, B. W., Strickler, J. E., Gorniak, J. G. and Debouck, C. (1989) *Biochem. Biophys. Res. Commun.* **159**, 420–425
- Schneider, J. and Kent, S. B. H. (1988) *Cell* **54**, 363–368
- Wlodawer, A., Miller, M., Jaskolski, M., Sathyanarayana, B. K., Baldwin, E., Weber, I., Selk, L., Clawson, K., Schneider, J. and Kent, S. B. H. (1989) *Science* **245**, 616–621
- Fitzgerald, P. M. D., McKeever, B. M., Van Middlesworth, J. F., Springer, J. P., Heimbach, J. C., Leu, C.-T., Herber, W. K., Dixon, R. A. F. and Drake, P. L. (1990) *J. Biol. Chem.* **265**, 14209–14219
- Swain, A. L., Miller, M. M., Green, J., Rich, D. H., Schneider, J., Kent, S. B. H. and Wlodawer, A. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 8805–8809
- DiGregorio, M., Pickering, D. S. and Chan, W. W.-C. (1988) *Biochemistry* **27**, 3613–3617
- Chan, W. W.-C. and Pfuetzner, R. A. (1993) *Eur. J. Biochem.* **218**, 529–534
- Pfuetzner, R. A. and Chan, W. W.-C. (1993) *Eur. J. Biochem.* **218**, 523–528
- Pfuetzner, R. A. and Chan, W. W.-C. (1988) *J. Biol. Chem.* **263**, 4056–4058
- Sheehan, J. C. and Hess, G. P. (1955) *J. Am. Chem. Soc.* **77**, 1067–1068
- König, W. E. and Geiger, R. (1970) *Chem. Ber.* **103**, 788–798
- Darke, P. L., Nutt, R. F., Brady, S. F., Garsky, V. M., Ciccarone, T. M., Leu, C.-T., Lumma, P. K., Freidinger, R. M., Veber, D. F. and Sigal, I. S. (1988) *Biochem. Biophys. Res. Commun.* **156**, 297–303
- Yonetani, T. and Theorell, H. (1964) *Arch. Biochem. Biophys.* **106**, 243–251
- Dixon, M. (1953) *Biochem. J.* **55**, 170–171
- Kempf, D. J., Norbeck, D. W., Codcovi, L., Wang, X.-C., Kohlbrenner, W. E., Wideburg, N., Paul, D. A., Knigge, M. F., Vasavanonda, S., Craig-Kennard, A., Saldivar, A., Rosenbrook, W. Jr., Clement, J. J., Plattner, J. J. and Erickson, J. (1990) *J. Med. Chem.* **33**, 2687–2689
- Schechter, I. and Berger, A. (1967) *Biochem. Biophys. Res. Commun.* **27**, 157–162
- Erickson, J., Neidhart, D. J., VanDrie, J., Kempf, D. J., Wang, X.-C., Norbeck, D. W., Plattner, J. J., Rittenhouse, J. W., Turon, M., Wideburg, N., Kohlbrenner, W. E., Simmer, R., Helfrich, R., Paul, D. A. and Knigge, M. (1990) *Science* **249**, 527–533
- Griffiths, J. T., Phylip, L. H., Konvalinka, J., Strop, P., Gustchina, A., Wlodawer, A., Davenport, R. J., Briggs, R., Dunn, B. M. and Kay, J. (1992) *Biochemistry* **31**, 5193–5200
- Poorman, R. A., Tomasselli, A. G., Heinrikson, R. L. and Kézdy, F. J. (1991) *J. Biol. Chem.* **266**, 14554–14561
- Cameron, C. E., Grinde, B., Jacques, P., Jentoft, J., Leis, J., Wlodawer, A. and Weber, I. T. (1993) *J. Biol. Chem.* **268**, 11711–11720
- Citri, N. (1973) *Adv. Enzymol.* **37**, 397–648
- Jencks, W. P. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4046–4050
- Shaw, E. (1970) in *The Enzymes* (Boyer, P. D., ed.), vol. 1, pp. 91–146, Academic Press, London and New York