Volume 219, number 1, 1–4 FEB 04884 July 1987

Discussion Letter

The mechanism of action of aspartic proteases involves 'push-pull' catalysis

László Polgár

Institute of Enzymology, Biological Research Center, Hungarian Academy of Sciences, PO Box 7, H-1502 Budapest, Hungary

Received 13 April 1987

In accord with the available kinetic and X-ray crystallographic data, it is proposed that the two catalytically competent carboxyl groups of aspartic proteases constitute a functional unit which mediates the proton from the attacking water molecule to the leaving nitrogen atom of the substrate. Protonation of this nitrogen atom has been the main issue of the previous mechanistic proposals. The first step of the present mechanism involves proton transfer from the water to the aspartic diad and concurrently another proton transfer from the diad to the carbonyl oxygen of the scissile peptide bond. These proton transfers provide the driving force for the bond formation between the substrate and water, which leads to the formation of a tetrahedral intermediate. The intermediate breaks down to products by a similar facilitation, i.e. by concerted general acid-base catalysis, which involves simultaneous proton transfers from the intermediate to the diad and from the diad to the leaving nitrogen of the substrate. The symmetrical mechanism of the formation and decomposition of the tetrahedral adduct resembles that found in the serine protease catalysis.

Pepsin; Aspartic protease; Enzyme mechanism

Aspartic proteases include several important enzymes, such as pepsin, chymosin, renin, cathepsin D and the proteases isolated from numerous fungi. The amino acid sequences of all these enzymes are homologous, in particular around the active-site residues. Comprehensive reviews on aspartic proteases have been published [1–3]. The three-dimensional structure of pepsin [4] and three microbial aspartic proteases including penicillopepsin [5,6], *Rhizopus chinensin* protease [7] and *Endothia parasitica* protease [7] has been reported. The tertiary structures of pepsin and the microbial enzymes show a striking similarity to each other. The substrate-binding cleft is large enough to accommodate polypeptides of about seven amino acid residues. This is consistent with the known specificity of aspartic proteases [1,3].

Correspondence address: L. Polgár, Institute of Enzymology, Biological Research Center, Hungarian Academy of Sciences, PO Box 7, H-1502 Budapest, Hungary

Two catalytically competent aspartyl residues are located deep in the centre of the cleft. The electron density maps suggest that the two carboxyl groups are hydrogen-bonded [6,8,9]. As indicated by pH-dependence studies, the two carboxyl groups must react in different forms, one in the ionized, and the other in the unionized form [10].

Transpeptidation reactions originally appeared to offer an explanation for the catalytic role of the aspartic residues [1]. Notably, it was assumed that transpeptidation proceeded either through an amino-enzyme or through an acyl-enzyme. The

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

former would contain a covalent peptide bond, the latter a covalent acid anhydride bond. The amino-enzyme was usually preferred to the acyl-enzyme. A covalent intermediate, however, could not be trapped, despite several attempts.

Fruton [11] was the first who questioned the formation of a covalent intermediate in the catalysis by aspartic proteases. Considerable evidence against the formation of covalent intermediate was provided by the studies of ^{18}O incorporation into transpeptidation products of the reactions carried out in $H_2{}^{18}O$-enriched water [12,13]. Recent NMR studies, indicating that water, rather than a carboxyl group, is the nucleophile, have provided additional evidence against covalent intermediate formation during catalysis by the aspartic proteases [14]. X-ray crystallographic studies have also suggested that the formation of a covalent acyl- or amino-enzyme is not feasible because of steric hindrance [8,15].

Since the covalent mechanism is not generally discounted [16,17], it is appropriate to point out a disadvantage of the formation of covalent intermediate. Specifically, the carboxyl group engaged in the bond formation would be lost as a catalyst although facilitation by both carboxyl groups, one as a general acid and one as a general base, is essential for the hydrolysis of an amino-enzyme.

The most detailed mechanism, involving the stereochemistry of the catalysis, has been reported recently [18]. This mechanism, which involves no covalent intermediate, is based on the highly refined crystal structure of penicillopepsin [6] and of its complex with a pepstatin fragment at 0.18 nm resolution [15]. Pepstatin is a potent transition-state analogue inhibitor of aspartic proteases, which can be strongly bound in the active-site cleft. The binding of substrate as deduced from the binding of pepstatin and a pepstatin analogue provides the clue as to how to place the scissile peptide bond with respect to the catalytic groups, Asp 32 and Asp 215 [8,15,18,19]. This binding mode indicates that it is Asp 32 (pepsin numbering) that catalyses the nucleophilic attack by the water molecule. Thus, in the enzyme-substrate complex Asp 32 would bear the negative charge and Asp 215 would be protonated. The hydrogen bond between the two carboxyl groups appears to prevail also in the enzyme-inhibitor complex [15] and presumably in the enzyme-substrate complex as well.

It was proposed [18] that the catalytic reaction is initiated by the protonation of the carbonyl oxygen of the substrate, which is in a good position to accept the proton from the carboxyl group of Asp 215. This is followed by the nucleophilic attack of a hydroxide ion generated from water by donating its proton to Asp 32. The breakdown of the resultant tetrahedral intermediate could be accomplished through two alternative pathways [18]. One involves a proton transfer mediated by Asp 215 from the protonated carbonyl oxygen atom of the tetrahedral intermediate (fig.1), the other involving protonation from the bulk water. In the following discussion the difficulties associated with the details of this mechanism are considered.

As for the formation of the tetrahedral intermediate, it is chemically unlikely that protonation of the substrate carbonyl oxygen would occur prior to the nucleophilic attack of the water molecule, as suggested [8,18]. The proton hydrogen-bonded between the two carboxyl groups, and held by the negative charge on them, cannot be transferred to the much less basic neutral peptide carbonyl oxygen atom without the assistance of the water attack. It is even more improbable that a hydroxide ion would be generated in the acidic media, where aspartic proteases usually operate. All the above problems are eliminated if we assume that protonation of the carbonyl oxygen and the base-catalysed nucleophilic attack are approximately simultaneous processes (fig.2a,b) as discussed later.

The breakdown of the tetrahedral intermediate

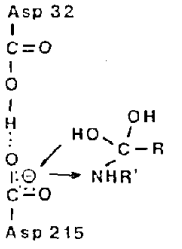

Fig.1. Stepwise proton transfers from the protonated carbonyl oxygen to the leaving nitrogen atom. The arrows show the proton transfer to Asp 215 and the subsequent transfer of the same proton to the leaving group, as suggested in [18].

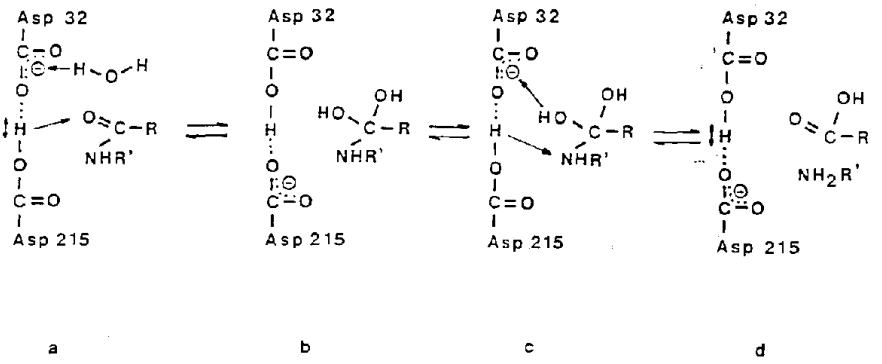

Fig.2. Proposed pathway for the catalysis by aspartic proteases. Proton transfers are indicated by arrows. They are concerted processes in forms a and c.

involves more uncertainties; two alternative proposals for its mechanism have been offered [18]. In one of the alternatives the source for protonation of the leaving nitrogen atom is the bulk solvent [18]. However, enzymes rarely use specific acid or specific base catalysis in their reactions. Protonation by bulk water, not aided by an enzymic group, is a typical specific acid catalysis. One may argue that aspartic proteases operate at low pH, where the concentration of the oxonium ion is sufficiently high for an effective specific acid catalysis. However, the principle of microscopic reversibility [20] prescribes that specific base catalysis, the reverse of specific acid catalysis, ought to facilitate peptide synthesis if the forward reaction is catalysed by oxonium ions. The reaction of hydroxide ions, however, could hardly be important at acidic pH, where peptide synthesis by pepsin takes place [21].

The other alternative of the leaving group protonation involves proton transfer from the carbonyl oxygen by the participation of Asp 215 [18]. In the first step of this mechanism the proton is transferred to the carboxylate ion of Asp 215 with the resultant formation of a negative tetrahedral adduct (cf. fig.1). This is followed by a general acid catalytic step, i.e. protonation of the leaving nitrogen atom by Asp 215. It should be pointed out that the first step of this mechanism may not be feasible for two reasons. (i) From the basic oxygen atom of the tetrahedral intermediate the proton cannot be transferred to the acidic carboxyl group in the absence of facilitation, such as the concurrent protonation of the leaving nitrogen atom. (ii) Simultaneous protonation of both carboxyl groups above pH 2 is inconsistent with the pK_a values of the carboxyl groups, which are less than 2 and between 4 and 5, respectively.

The catalytic pathway proposed below (fig.2) overcomes the problems of the previously suggested mechanisms and is consistent with all experimental data, including those pertinent to the stereochemistry of the catalysis. In the proposed mechanism the two catalytically competent carboxyl groups constitute a functional unit, like the catalytic triad and the thiolate-imidazolium ion pair in the catalyses by serine and cysteine proteases, respectively [22]. The extraordinarily symmetrical structural details around the two carboxyl groups [6,9] suggest that the diad proton may be bound covalently to either carboxyl group in the free enzyme. In accord with the symmetry of the diad, it is proposed that formation and decomposition of the tetrahedral intermediate are symmetrical processes. Two concurrent proton transfers taking place in both the formation and breakdown of the tetrahedral intermediate are the essential features of the catalysis. When the substrate is bound properly with respect to the catalytic groups, the proton of the attacking nucleophile is accepted by the diad carboxylate ion, while the diad proton is donated to the carbonyl oxygen of the substrate (fig.2a,b). Possible concertedness of the proton transfers, leading to hydroxide ion formation, has already been proposed [8]. By contrast, no hydroxide ion is generated in the present mechanism since the proton donation to the carboxyl group is coupled with the nucleophilic attack on the carbonyl carbon by water. This represents a true general base catalysis,

which precludes the formation of high-energy intermediates. The simultaneous proton transfers portray a 'push-pull' general acid-base catalysed nucleophilic attack [23]. The breakdown of the resultant adduct proceeds by the same mechanism (fig.1c,d). The proton from the protonated oxyanion is transferred to the diad, while the diad proton protonates the leaving nitrogen with the concurrent C–N bond cleavage. In this mechanism the protonation of the nitrogen atom takes place not from the protonated oxyanion as has been previously proposed [18] and shown in fig.1, but the proton stems from the nucleophile and is conveyed to the leaving group by the diad. Thus, the mechanism is symmetrical for the formation and breakdown of the tetrahedral intermediate, as both processes are facilitated by push-pull general acid-base catalyses.

The symmetry principle emphasized in the above mechanism is also characteristic of the catalysis of serine proteases [22]. It is a remarkable advantage of the symmetry of catalysis that a single functional unit effects both bond making and bond breaking. The mechanism proposed here for aspartic proteases and that of serine proteases are also similar in that the protonation of the leaving group takes place from the nucleophile in both cases, with the help of the diad of the two carboxyl groups and a histidine residue, respectively. However, in serine protease catalysis a single proton is transferred, whereas in the case of aspartic proteases two concurrent proton transfers promote the formation of the tetrahedral adduct, as well as its breakdown.

ACKNOWLEDGEMENT

The author is indebted to Professor J.S. Fruton of Yale University for sending his review article before publication.

REFERENCES

[1] Fruton, J.S. (1971) in: The Enzymes, vol.3, 3rd edn (Boyer, P.D. ed.) pp.119–164, Academic Press, New York.
[2] Tang, J. (1979) Mol. Cell. Biochem. 26, 93–109.
[3] Fruton, J.S. (1987) in: New Comprehensive Biochemistry (Neuberger, A. and Brocklehurst, K. eds) chap.1, Elsevier, Amsterdam, New York, in press.
[4] Andreeva, N.S., Zdanov, A.S., Gustchina, A.E. and Fedorov, A.A. (1984) J. Biol. Chem. 259, 11353–11364.
[5] Hsu, I.-N., Delbaere, L.T.J., James, M.N.G. and Hofmann, T. (1977) Nature 266, 140–145.
[6] James, M.N.G. and Sielecki, A.R. (1983) J. Mol. Biol. 163, 299–361.
[7] Subramanian, E., Swan, I.D.A., Liu, M., Davies, D.R., Jenkins, J.A., Tickle, I.J. and Blundell, T.L. (1977) Proc. Natl. Acad. Sci. USA 74, 556–559.
[8] Bott, R., Subramanian, E. and Davies, D.R. (1982) Biochemistry 21, 6956–6962.
[9] Pearl, L. and Blundell, T. (1984) FEBS Lett. 174, 96–101.
[10] Clement, G.E. (1973) in: Prog. Bioorg. Chem., vol.2 (Kaiser, E.T. and Kézdy, F.J. eds) pp.177–238, Wiley, New York.
[11] Fruton, J.S. (1976) Adv. Enzymol. 44, 1–36.
[12] Antonov, V.K., Ginodman, L.M., Kapitannikov, Yu.V., Barshevskaya, T.N., Gurova, A.G. and Rumsh, L.D. (1978) FEBS Lett. 88, 87–90.
[13] Antonov, V.K., Ginodman, L.M., Rumsh, L.D., Kapitannikov, Yu.V., Barshevskaya, T.N., Yavashev, L.P., Gurova, A.G. and Volkova, L.I. (1981) Eur. J. Biochem. 117, 195–200.
[14] Schmidt, P.G., Holladay, M.W., Salituro, F.G. and Rich, D.H. (1985) Biochem. Biophys. Res. Commun. 129, 597–602.
[15] James, M.N.G., Sielecki, A.R., Salituron, F., Rich, D.H. and Hofmann, T. (1982) Proc. Natl. Acad. Sci. USA 79, 6137–6141.
[16] Hofmann, T., Dunn, B.M. and Fink, A.L. (1984) Biochemistry 23, 5247–5256.
[17] Spector, L.B. (1982) Covalent Catalysis by Enzymes, pp.137–142, Springer, New York.
[18] James, M.N.G. and Sielecki, A.R. (1985) Biochemistry 24, 3701–3713.
[19] Hofmann, T., Hodges, R.S. and James, M.N.G. (1984) Biochemistry 23, 635–643.
[20] Jencks, W.P. (1969) Catalysis in Chemistry and Enzymology, pp.613–614, McGraw-Hill, New York.
[21] Pellegrini, A. and Luisi, P.L. (1978) Biopolymers 17, 2573–2580.
[22] Polgár, L. and Halász, P. (1982) Biochem. J. 207, 1–10.
[23] Jencks, W.P. (1969) Catalysis in Chemistry and Enzymology, p.200, McGraw-Hill, New York.