KINETIC EVIDENCE FOR TWO DIFFERENT PRODUCTIVE SUBSTRATE BINDING SITES IN SUBTILISINS

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1. Introduction

X-ray diffraction studies of subtilisin BPN'/inhibitor complexes revealed two different binding modes for several reversible and irreversible inhibitors. The peptide chloromethyl ketones, e.g. Z–Ala–Gly–Phe–CH_2Cl*, form one class of irreversible inhibitors [1, 2], and the hydrophobic binding site of these inhibitors on subtilisin BPN' has been labelled the A-site [3]. The second class consists of the reversible inhibitor BA, the acylating agent TCI and phenylmethanesulfonylfluoride, all of which have been shown to bind to the so-called B site of S. BPN' crystals [3]. It has been suggested recently that the B site could be a second productive substrate binding site in S. BPN' [3, 4].

The following data offer kinetic evidence for two productive substrate binding sites in S. Novo and S. Carlsberg. Cleavage of Ala–Ala–Phe–OMe and Ac–Ala–Ala–Phe–Ala is inhibited competitively by ZAAP. BA is a non-competitive** inhibitor for the same reaction. Cleavage of TCI by S. Novo is competitively inhibited by BA, but non-competitively by ZAAP. This behavious is consistent with the assumption that peptide substrates and ZAAP bind at the crystallographically defined A-site, while TCI and BA bind in the B-site. Identical results are obtained for S. Carlsberg except that BA is a competitive inhibitor towards all the substrates tested. Cleavage of BAE by S. Novo and S. Carlsberg is inhibited non-competitively by BA and ZAAP.

2. Materials and methods

Purified S. Novo and S. Carlsberg (products of Novo Industires, Copenhagen), were kindly provided by R.S. Roche. The commercial enzymes were chromatographed consecutively on Sephadex G-25 and on CM-cellulose as outlined elsewhere [2]. Ac–Ala–Ala–Ala–Phe–Ala was a gift from Dr. H. Benderly. Z–Ala–Ala–Phe–OMe and Z–Ala–Ala–Phe were prepared by coupling Z–Ala–Ala–ONSu with phenylalanine methyl ester and phenylalanine, respectively [5]. The carbobenzoxy group was removed by HBr in acetic acid, and the hydrobromide HBr. H–Ala–Ala–Phe–OMe was recrystallized from 1-butanol – ether. ZAAP was recrystallized from ethyl acetate – hexane. TCI was synthesized according to the literature [6]. BA, BAE and ATE were prepared by standard procedures in our laboratory.

Esterase and peptidase activity were followed by the pH stat method (Radiometer titration assembly) in 0.1 M KCl at pH 8.6 and 37°. The deacylation of cinnamoylsubtilisins at 25° and pH 7 (0.04 M phosphate buffer, I = 0.1 with KCl, 3.3% (v/v) acetonitrile) was monitored at 335 nm on a Gilford 2400-S spectrophotometer according to 'method A' of

* Abbreviations according to IUPAC-IUB rules, J. Biol. Chem. 247 (1972) 977. Further abbreviations are ZAAP, benzyloxycarbonylalanyl-alanyl-phenylalanine; BA, N^{α}-benzoylarginine ethyl ester; ATE, *N*-acetyltyrosine ethyl ester; TCI, *N*-*trans*-cinnamoylimidazole; S., subtilisin.

** Throughout this paper 'non-competitive' means that substrate and inhibitor do not compete for the same site.

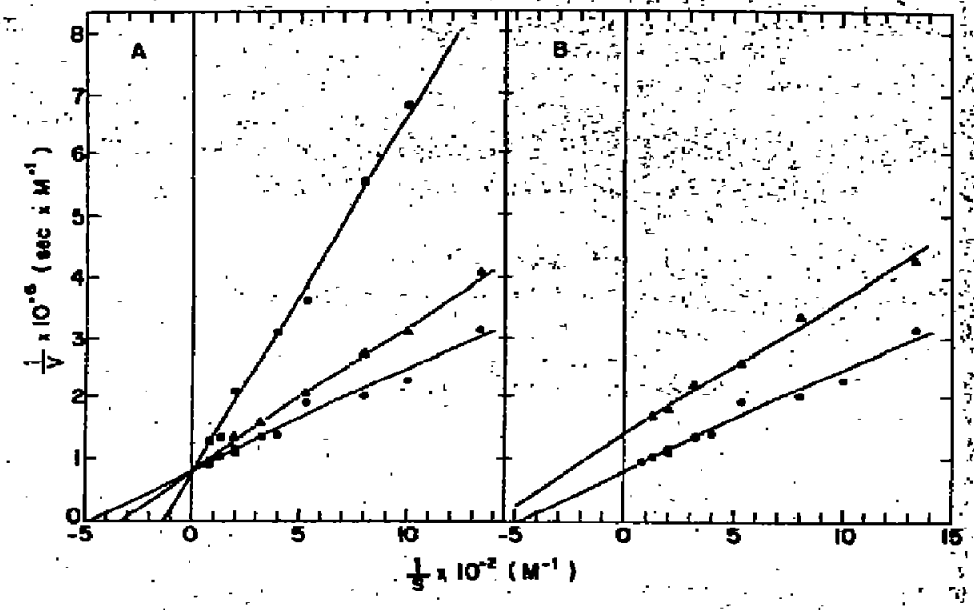

Fig. 1. Hydrolysis of Ala–Ala–Phe–OMe by subtilisin Novo at pH 8.6 and 37° in 0.1 M KCl. Velocity v is expressed in moles of substrate cleaved per ℓ and per sec. • No inhibitor. A) ▲ 1 mM ZAAP; ■ 5 mM ZAAP. B) ▲ 20 mM BA.

Bender et al. [7]. An extinction coefficient of 9.04 × 10^3 M at 335 nm was used for TCI [7]. Appropriate enzyme stock solutions were prepared in water (pH stat assay) or in 0.05 M acetate, pH 5.5 (spectroscopic assay). Substrate and inhibitor stock solutions were 0.05 M to 0.01 M in 0.1 M KCl. TCI stock was 3 × 10^{-3} M in acetonitrile.

The Lineweaver–Burk plots were obtained from a weighted least square analysis of the experimental points (Hewlett-Packard 9820 A calculator).

Descending paper chromatography was done on Whatman no. 1 sheets with the upper phase of a mixture of 1-butanol–acetic acid–water (25:6:25) as mobile phase.

3. Results

Figs. 1 to 4 show Lineweaver–Burk plots of the experiments with S. Novo. Esterase and peptidase activity of S. Novo were inhibited competitively by ZAAP (figs. 1A and 2A). BA was a non-competitive inhibitor for the same two reactions (figs. 1B and 2B). Towards TCI as a substrate the same two inhibitors changed their character in the way that ZAAP became a non-competitive and BA became a competitive inhibitor (fig. 3). Neither of the two inhibitors behaved competitively towards BAE (fig. 4). As for S. Carlsberg, inhibition of TCI hydrolysis was similar to the results with S. Novo (fig. 5). ZAAP was a competitive inhibitor when tested against the peptide ester Ala–Ala–Phe–OMe or ATE, but BA continued to behave competitively (figs. 6 and 7).

The cleavage of Ac–Ala–Ala–Phe–Ala by S. Novo

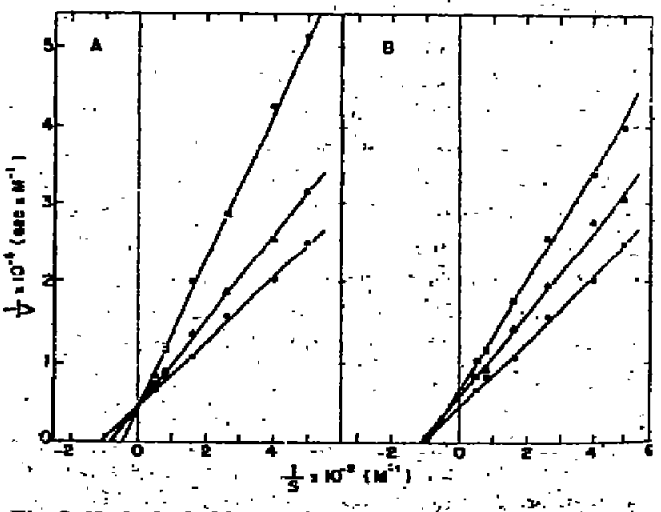

Fig. 2. Hydrolysis of Ac–Ala–Ala–Phe–Ala by subtilisin Novo. Conditions as in fig. 1. Velocity v is expressed in moles of OH^- consumed per ℓ and per sec. • No inhibitor. A) ▲ 2 mM ZAAP; ■ 5 mM ZAAP. B) ▲ 20 mM BA; ■ 37.5 mM BA.

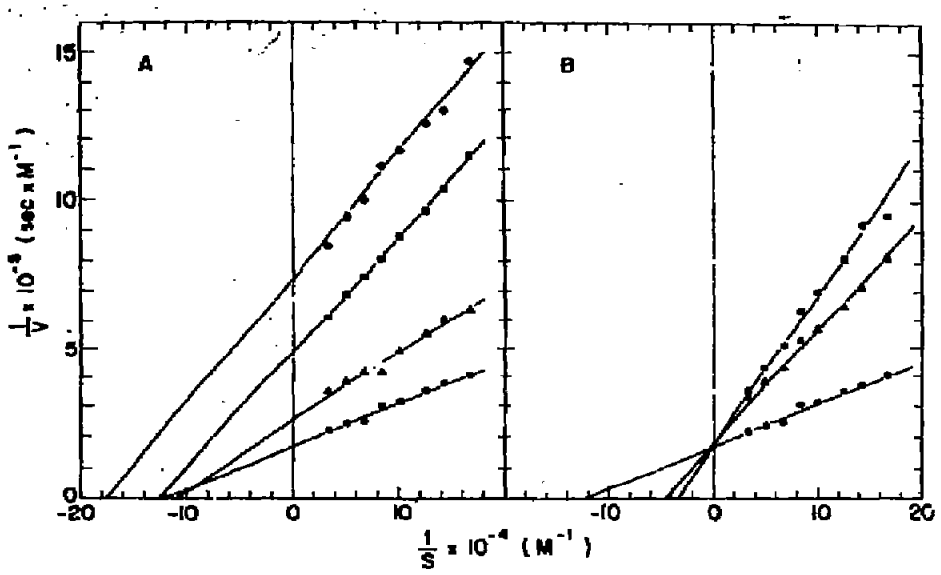

Fig. 3. Hydrolysis of *N-trans*-cinnamoylimidazole by subtilisin Novo at pH 7.0 and 25°. Velocity r is expressed as in fig. 1. • No inhibitor. A) ▲ 0.5 mM ZAAP; ■ 2 mM ZAAP; ● 5 mM ZAAP. B) ▲ 10 mM BA; ■ 20 mM BA.

was analyzed qualitatively by paper chromatography. The only ninhydrin positive fragments detected were free alanine and Phe–Ala in about equal amounts. Thus cleavage occurred at either the first or the second bond from the carboxyl end.

4. Discussion

The peptides Ala–Ala–Phe–OMe or Ac–Ala–Ala–Phe–Ala and ZAAP evidently compete for the same site on both subtilisins as indicated by their strictly competitive behaviour towards each other (figs. 1A, 2A, 6 and 7). As for S. Novo which is identical with S. BPN' [8, 9] the binding site for the above three peptides is probably identical with that found for the peptide chloromethyl ketones from X-ray analysis [1, 2]. This site was defined as the A site [3]. The similarity in sequence of the peptides in the present study and the chloromethyl ketones used by Kraut and coworkers strengthens this assumption. BA clearly does not compete for the A site on S. Novo but can form the ternary complex enzyme/substrate/inhibitor (figs. 1B and 2B). In the S. BPN' crystal BA, indeed, binds at a locus labelled B site which is clearly separated from the A site [3, 4]. Assuming the same conformation for the Novo enzyme in solution BA binds to the crystallographically defined B site in the inhibition experiments.

The inhibition pattern changes completely when TCI is the substrate (fig. 3). BA clearly becomes a competitive inhibitor, whereas ZAAP can now form a ternary complex. Competitiveness of BA against TCI has been observed recently by Wright [3]. The straightforward interpretation of this result is that TCI binds to a different site than does the peptide substrate, and

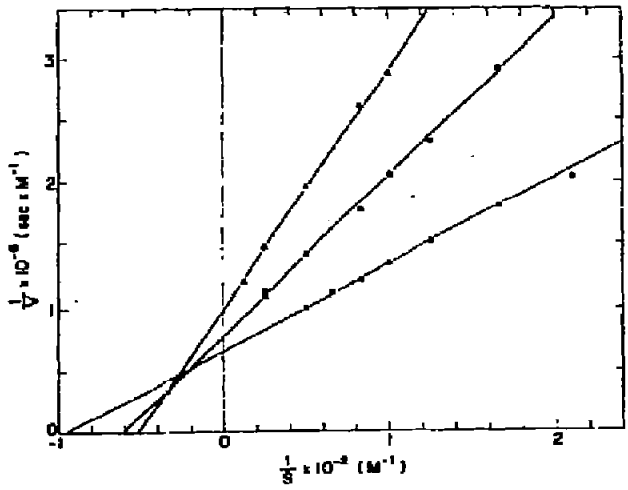

Fig. 4. Hydrolysis of N^{α}-benzoylarginine ethyl ester by subtilisin Novo. Conditions as in fig. 1. • No inhibitor; ▲ 5 mM ZAAP; ■ 20 mM BA.

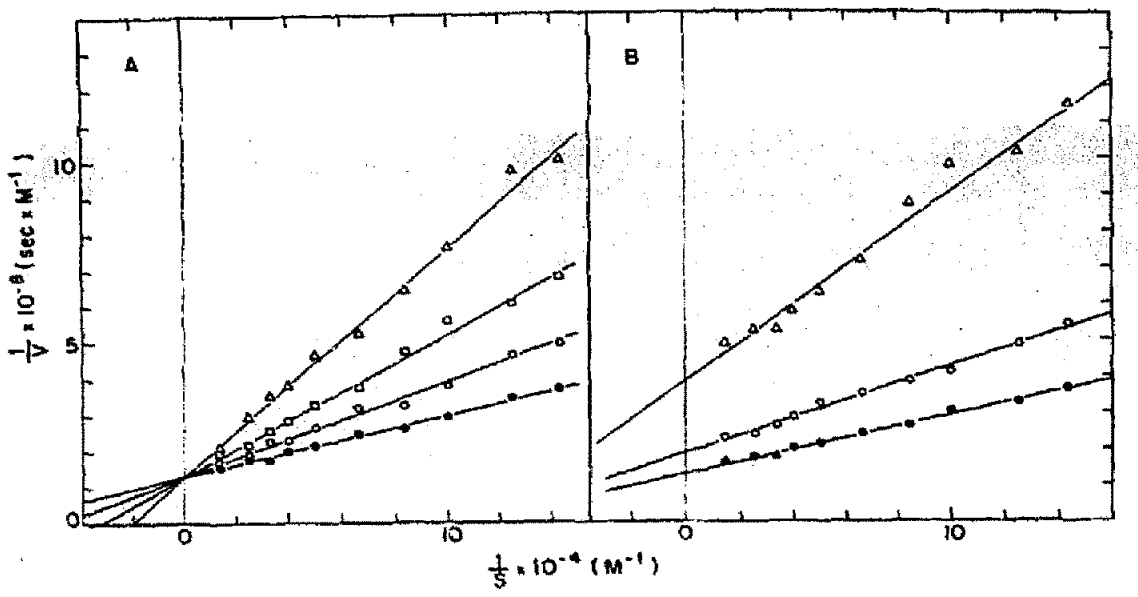

Fig. 5. Hydrolysis of *N-trans*-cinnamoylimidazole by subtilisin Carlsberg. Conditions as in fig. 3. • No inhibitor. A) ○ 10 mM BA; □ 20 mM BA; △ 40 mM BA. B) ○ 0.1 mM ZAAP; △ 0.5 mM ZAAP.

probably at the same site as BA. However, since in TCI hydrolysis deacylation is rate limiting [7], the competitive inhibition pattern with BA need not rule out a ternary complex of the form enzyme/TCI/BA. If in the reaction scheme

$$E \cdot BA + TCI \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot BA \cdot TCI \xrightarrow{k_2} ETC \xrightarrow{k_3} E + TC$$
$$\downarrow BA, im$$

im = imidazole; TC = *trans*-cinnamic acid

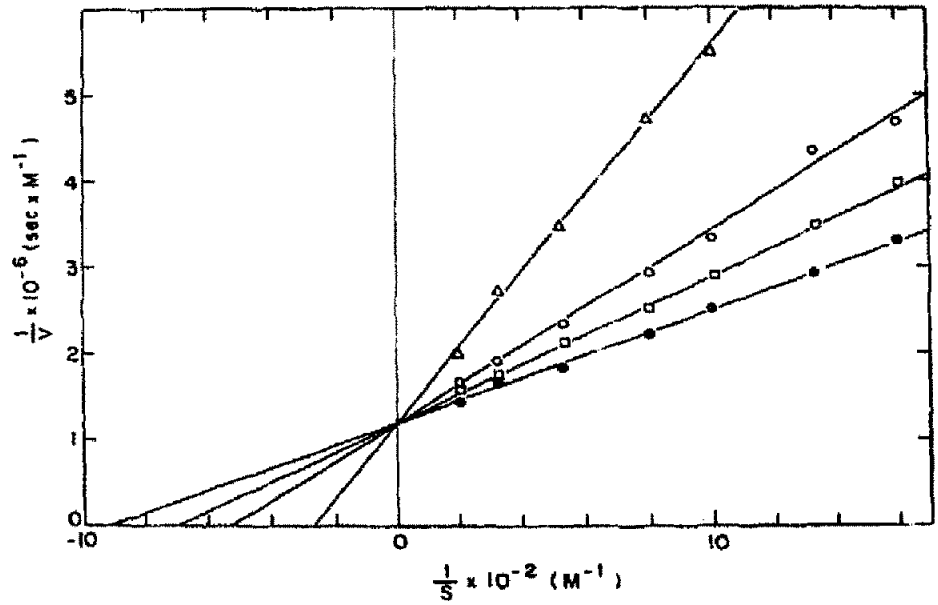

Fig. 6. Hydrolysis of Ala–Ala–Phe–OMe by subtilisin Carlsberg. Conditions as in fig. 1. • No inhibitor; △ 2 mM ZAAP; □ 0.1 mM ZAAP; ○ 20 mM BA.

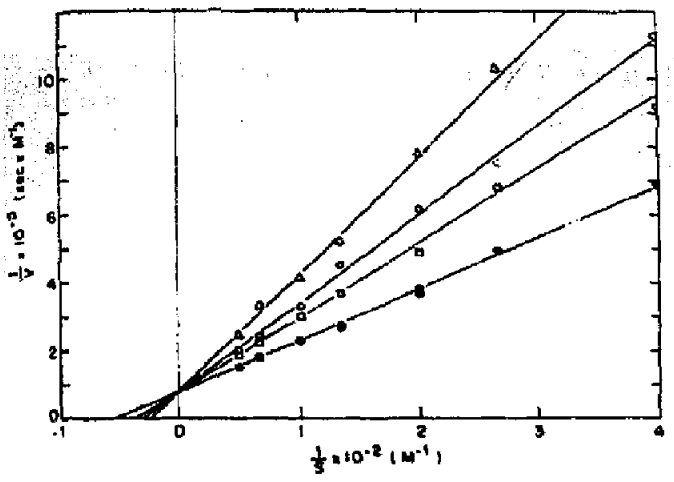

Fig. 7. Hydrolysis of *N*-acetyltyrosin ethyl ester by subtilisin Carlsberg. Conditions as in fig. 1. ● No inhibitor; ▲ 1 mM ZAAP; ▫ 18 mM BA; ○ 36 mM BA.

k_3 is rate limiting, competitive inhibition will be observed despite different binding sites for BA and TCI. Once the acylenzyme (ETC) is formed, BA can no longer bind to the enzyme, otherwise inhibition would be non-competitive (reduced k_3). X-ray evidence is compatible with this conclusion. The cinnamoyl moiety in the crystalline acylenzyme (S. BPN') occupies the same place as BA [3]. Each enzyme clearly has only a single catalytic site [10], and both productive binding sites must accomodate their substrate so that the bond to be cleaved is favourably oriented to the single catalytic site.

Hydrolysis of BAE by S. Novo is inhibited non-competitively by ZAAP and BA (fig. 4). This could be interpreted as follows: BAE binds productively either in site A or in site B but not in both sites simultaneously. BA binds in site B only. A ternary complex of the enzyme with BA and BAE is possible only if BAE binds to the A site. The steady state kinetics of this system would show Michaelis–Menten behaviour and therefore allow for the straight lines in fig. 4. The same reasoning stands also for ZAAP bound to site A and taking part in a ternary complex with BAE bound to site B. Alternatively, BAE could yet show a third productive binding mode. Different productive binding modes for BAE and ATE were also proposed by Glazer [11] who found that ATE hydrolysis but not BAE hydrolysis is inhibited competitively by hydrocinnamate and indole.

Results with S. Carlsberg are identical with those from Novo enzyme except for the inhibition by BA. BA behaves competitively against all the substrates tested (figs. 5 to 7). One possible explanation is that BA at least partly covers both substrate binding sites on the Carlsberg enzyme. This would imply that the A and B sites have a somewhat different topography in comparison with S. Novo. S. Carlsberg differs from S. BPN' (or S. Novo) in only 84 out of 274 amino acid positions [12]; and the active site regions of the two homologous enzymes seem to share many common features [10, 12]. However, the different inhibition behaviour of BA towards the two enzymes reported here points to some subtle differences in the two active sites. Differences in the geometry of the active sites of S. Novo and S. Carlsberg have also been deduced from fluorescence experiments [13].

It has been suggested that the two substrate binding sites might contribute to the low specificity requirements of the subtilisins [3, 4]. With the possible exception of BAE the few peptide substrates tested here all seem to bind to the A site. It is therefore highly probable that the A site constitutes the important binding site for both larger and smaller substrates and that only in some rare cases (charged substrates have been suggested [1, 4]) a different productive binding mode may prevail.

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