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KINETICS AND MECHANISM OF PROTEINASE-BINDING OF PREGNANCY ZONE PROTEIN (PZP). APPEARANCE OF SULFHYDRYL GROUPS IN REACTIONS WITH PROTEINASES

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Proteinase binding by pregnancy zone protein (PZP), an α-macroglobulin involves bait region cleavages, association of dimeric-PZP into tetrametic and reaction of internal γ-glutamyl-β-cysteinyl thiol esters of PZP with proteinase side chains. The product is an equimolar enzyme-PZP(tetramer) covalently linked complex with four free sulfhydryl groups. The kinetics of the appearances of sulfhydryl groups during the reaction of PZP with chymotrypsin has been investigated using stopped-flow and conventional mixing techniques over a broad concentration range. Thiol ester cleavages followed double exponential decays corresponding with two steps. The faster one resulted in the appearance of three sulfhydryl groups with an observed rate constant, $k_{obs} = k_{1,1} + k_{1,2}\,\Delta E$, dependent on the excess concentration of chymotrypsin, ΔE, and $k_{1,1} = 0.03\,s^{-1}$ and $k_{1,2} = 4 \cdot 10^4\,M^{-1}s^{-1}$. The last sulfhydryl group appeared in a slower step, with similar concentration dependence and $k_{2,1} \sim 0.003\,s^{-1}$ and $k_{2,2} \sim 5 \cdot 10^3\,M^{-1}s^{-1}$. Covalent binding of the enzyme apparently was simultaneous with the faster thiol ester cleavage step.

Based on these and previous results a model of the reaction mechanism of the proteinase binding reaction of PZP is proposed. It consists of four major steps: (i) Bait region cleavage of PZP-dimers by the enzyme, (ii) fast association of enzyme-PZP(dimer) species with native PZP or with another enzyme-PZP(dimer) compound resulting in release of one of the associated enzyme molecules (iii) reaction of an average of three thiol esters of the enzyme-PZP(tetramer) intermediate with the associated internal enzyme molecule or with an external one. In this step one enzyme molecule becomes covalently linked to the PZP-(tetramer), three sulfhydryl groups appear and the enzymic activity of the bound enzyme molecule decreases to the level of that of the final complex. (iv) Hydrolysis of the last thiol ester and in the presence of excess enzyme, degradation of enzyme-PZP(tetramer) complexes and formation of fragments some of which are the size of PZP(dimer) with enzyme bound.

KEY WORDS: Pregnancy Zone Protein; α_2-macroglobulin, chymotrypsin; stopped-flow kinetics, thiol ester reactions.

INTRODUCTION

Human pregnancy zone protein (PZP) is a pregnancy associated dimeric plasma protein (360 kDa), which belongs to the α-macroglobulin family (see[1] for a review). Using chymotrypsin as a model enzyme the binding reaction has been shown to

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Abbreviations: PZP, pregnancy zone protein; α_2M, α_2-macroglobulin; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Suc-Ala-Ala-Pro-Phe-pNA, succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenyl-alanine-4-nitroanilide.

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involve proteolysis of critical peptide segments (the bait regions) of PZP, dimerization of 360-kDa PZP-dimers into PZP-tetramers, cleavage of internal PZP-β-cysteinylγ-glutamyl thiol ester bonds and covalent binding of the proteinase. The reaction results in the formation of equimolar covalently bound enzyme-PZP(tetramer)-complexes.[2,3]

Kinetic studies of the changes of intrinsic protein fluorescence of the proteinase-binding reaction of PZP using chymotrypsin have indicated that the reaction proceeds via an enzyme-PZP(tetramer) intermediate with intact thiol ester groups.[4] In the present paper we report the results of stopped-flow kinetic studies of the appearance of free sulfhydryl groups of PZP and of the decrease of the enzymic activity of the proteinase in the PZP-chymotrypsin reaction. Based on these and previous results[3,4] a model of the reaction mechanism of the PZP-proteinase binding reaction is presented.

MATERIALS AND METHODS

Proteins

PZP was prepared from late pregnancy serum as described by Sand *et al.*[2] The serum was obtained through the courtesy of the staff at the Department of Gynaecology, Aarhus University Hospital. Freshly prepared PZP in 0.1 M Na_2HPO_4/NaH_2PO_4, pH 8.0, contained only small amounts of 720-kDa tetramers and no aggregates of high molecular mass as judged from nondenaturing PAGE and from absorbance measurements at 280 nm ($E_1^{1\%} = 8.2$[2]) before and after filtering through 0.47 μm membrane filters. Aggregation inevitably occurred when PZP-preparations were stored for long periods and batches containing aggregated material gave non-reproducible results. Typically, PZP-preparations were stored at 0°C and were stable for 1–3 months.[3] The concentration of active PZP of the stock solution was determined from DTNB-titrations of the sulfhydryl groups appearing in reactions of PZP with methylamine and with chymotrypsin of known active concentration as described previously.[3] The titrations of the stock solution was 78 $\pm$ 2% of that obtained from absorbance measurements at 280 nm, showing 2.8 μM active PZP and 3.6 μM total PZP. The PZP-concentrations are given in units of tetrameric PZP corresponding to the molar amount equivalent to that of one PZP(tetramer)-chymotrypsin complex.[3,4]

Bovine α-chymotrypsin was from BDH, Poole, U.K. Stock solutions (10–100 μM) in 10^{-3} M HCl, pH 3 were kept in aliquots at −20°C. Their active site concentrations were determined by titrations with soybean trypsin inhibitor (BHD, Poole, U.K.), the active concentration of which was determined using 4-nitrophenyl-4′-guanidino-benzoate-titrated trypsin; a method analogous to that described by Ganrot.[5]

Chemicals

Suc-Ala-Ala-Pro-Phe-pNA was from Bachem, Budendorf, Switzerland, DTNB, methylamine and other chemicals were analytical grade from Fluka, Buchs, Switzerland or from Merck, Darmstadt, Germany.

Experimental Procedures

The time courses of sulfhydryl group appearance in the reaction of PZP with chymotrypsin was followed by measuring the change in absorbance at λ = 412 nm in the

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presence of a large excess of DTNB (0.5 mM) at 25°C in 0.1 M Na_2HPO_4/NaH_2PO_4-buffer, pH 7.6 Some series of experiments were performed using manual mixing and Perkin-Elmer λ17spectrophotometer equipped with PECSS software for data acquisition and handling essentially as previously described for reactions with α_2-M.[6] Other series of experiments were performed using the stopped-flow method and a Hi-Tech Scientific PQ/SJ-53 instrument. PZP (1.5, 0.75, 0.38 μM) was reacted with chymotrypsin (0, 0.35, 0.7, 1.0, 1.7, 5.5, 14 and 28 μM, final concentrations).

To control the validity of the coupled assay method the reactions of DTNB with PZP-enzyme and PZP-methylamine complexes were also studied using the stopped-flow method. Reaction-mixtures of methylamine (320 mM) and PZP (1.5 μM) were incubated for 30 min at pH 7.6, 25°C, and of chymotrypsin (1.7 μM) and PZP (1.5 mM) for 10 min before mixing with DTNB (1, 0.5 and 0.25 mM, final concentrations).

In each stopped-flow experiment 400 pairs of data were recorded at equal time intervals. The sets of data from 4–5 experiments at identical conditions were averaged and were fitted to a number of non-linear analytical equations, e.g., those corresponding to first and second order kinetics and double exponential decays, using the Hi-Tech HS-1 Data Pro software. The non-linear regression analysis is based on the Gauss–Newton procedure.

Rates of hydrolysis of Suc-Ala-Ala-Pro-Phe-pNA catalysed by non-equilibrium mixtures of chymotrypsin and PZP were determined as a function of the PZP-chymotrypsin reaction time after manual mixing. Series of experiments were performed by incubating a fixed amount of chymotrypsin (68, 34 and 17 nM) with 8 different concentrations of PZP (0–5 times that of chymotrypsin) and measuring the initial steady state rate of hydrolysis of the substrate after 0.5, 1, 2, 3, 6, 9, 15, 45, 90 and 180 min of incubation. The concentration of Suc-Ala-Ala-Pro-Phe-pNA was 138 μM in the assay reaction mixture, that is ≈ 3 times K_m of free chymotrypsin and ≈ 2 times K_m of PZP-bound chymotrypsin.[3]

RESULTS

The reactions of DTNB with the thiol groups of PZP-complexes were investigated using the stopped-flow method at 1, 0.5 and 0.25 mM DTNB, and two such traces are illustrated in Figure 1. The reactions showed simple (pseudo) first-order behaviour and a rectilinear dependence of the pseudo-first-order constant, k_{obs}, on the concentration of DTNB (Figure 1 (insert)). The resulting values of the second-order rate constants of the sulfhydryl groups of the methylamine-PZP(dimer) (P_2) and the chymotrypsin-PZP(tetramer)-complexes (P_4) (see also below) with DTNB were not identical:

$$k_{P_4} = 6.4\ 10^4\ M^{-1}\ s^{-1}$$

$$k_{P_2} = 1.1\ 10^5\ M^{-1}\ s^{-1}$$

Apparently the association of PZP-dimers into tetramers, which occurs only in the reaction with enzymes,[3] slightly changes the reaction conditions of sulfhydryl groups and makes the reaction with DTNB slower. The value of the pseudo-first order rate constant that is relevant in our conditions at DTNB = 0.5 mM is $33\ s^{-1}$.

The kinetics of the appearance of sulfhydryl groups in the reaction of PZP and chymotrypsin was investigated using a coupled DTNB-assay. As discussed earlier[7/8,9,10]

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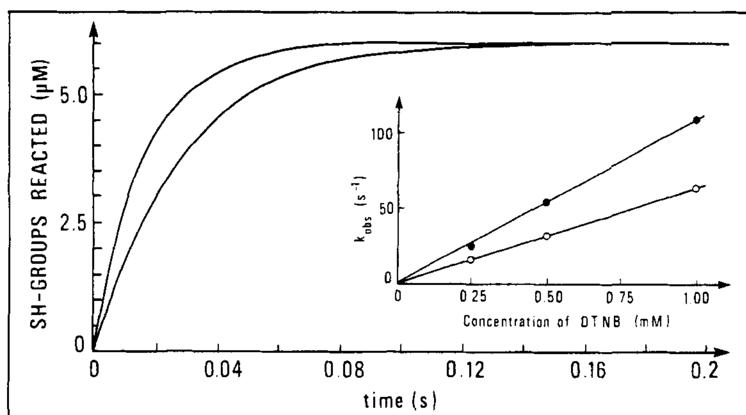

FIGURE 1 Reactions of DTNB with sulfhydryl groups of pre-reacted PZP-species. Upper curve: PZP-methylamine and lower curve: PZP-chymotrypsin. Experiments were performed in a stopped-flow apparatus with initial concentrations of DTNB (0.25, 0.5 (illustrated) and 1.0 mM) and of pre-reacted PZP (1.5 μM (molar unit: tetrameric PZP)). Insert: The dependence of the observed pseudo first-order rate constant, k_{obs} on the concentration of DTNB. Methylamine pre-reacted PZP (●) and chymotrypsin pre-reacted PZP (○).

the appearance of sulfhydryl groups in α-macroglobulins during complex formation with proteinases is multiphasic. Series of experiments were performed using manual mixing and stopped-flow mixing in order to obtain data on the slow and the fast phase, respectively, of the reaction. For all the experiments the time courses obtained of the appearance of sulfhydryl groups in the reaction of PZP with chymotrypsin were slow compared to that of the DTNB-reaction used for detection. The results of some typical experiments are shown in Figures 2 and 3, which illustrate manual and stopped-flow mixing experiments, respectively. Equations describing first-order, second-order and double exponential decays of the residual thiol ester groups, S_R, were fitted to the data. In all cases only double exponential decays showed satisfactorily goodness of fit (Eq. 1):

$$S_R/(P_4E)_\infty = (A_1 e^{-k_{1,obs}t} + A_2 e^{-k_{2,obs}t}) \quad (1)$$

where S_R is the concentration of residual thiol ester groups at time, t, and $(P_4E)_\infty$ is the concentration of the equimolar PZP-tetramer-chymotrypsin-complex ultimately formed in the actual experiment.

The concentration dependencies of the four kinetic parameters, A_1, A_2 ($A_1 + A_2 = 4$), $k_{1,obs}$ and $k_{2,obs}$ obtained from fits to Eq. (1) were investigated. The amplitudes, A_1 and A_2 were reasonably constant, $A_1 = 3$ and $A_2 = 1$. As is illustrated in Figure 4, A_1-values were in the range 2.8–3.2 and A_2-values, thus, 0.8–1.2. The concentration dependencies of $k_{1,obs}$ and $k_{2,obs}$ are illustrated in Figures 5 and 6, respectively. The results indicate a dissymmetry in the cleavages of the thiol-esters of tetrametic PZP-chymotrypsin intermediates. One apparently is cleaved slowly, whereas the other three are cleaved much faster and are not discernable.

Analysis of the concentration dependence of $k_{1,obs}$ (Figure 5) shows a good fit to Eq. (2):

$$k_{1,obs} = k_{1,1} + k_{1,2}\,(\Delta E) \quad (2)$$

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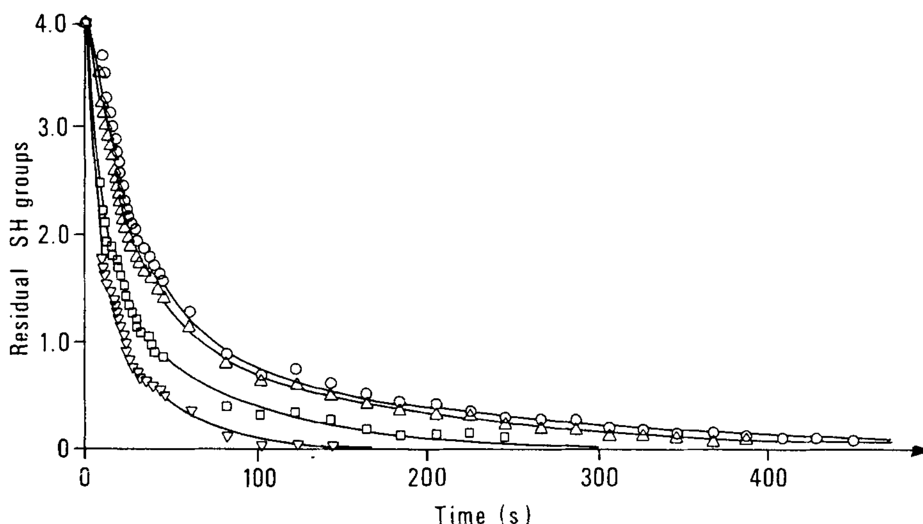

FIGURE 2 The decay of residual sulfhydryl groups to appear in reactions of PZP with chymotrypsin. A series of time courses obtained after manual mixing is shown. The concentrations were: PZP (1.5 μM (molar unit: tetrameric PZP)) and chymotrypsin (○) 0.7 μM; (△) 1 μM; (□) 1.7 μM and (▽) 5.5 μM. The solid lines are those given by the fitted double exponential equation (Eq. 1).

where ΔE is the concentration of chymotrypsin in excess of that necessary for forming a 1 : 1 complex, $k_{1,1}$ is a first order and $k_{1,2}$ a second order rate constant. The best fit results in $k_{1,1} = 0.03\,s^{-1}$ and $k_{1,2} = 4 \cdot 10^4\,M^{-1}s^{-1}$. More simple expressions predicting $k_{1,obs}$ to be constant or proportional to the chymotrypsin concentrations were tested, but were not in agreement with the results obtained.

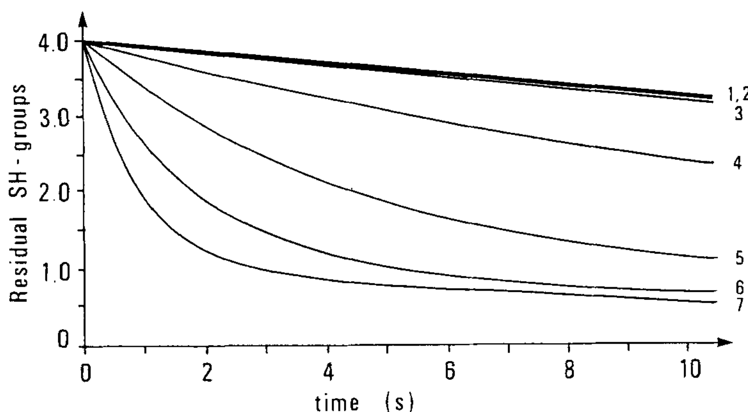

FIGURE 3 The decay of residual sulfhydryl groups to appear in reactions of PZP with chymotrypsin. One of the series of time courses obtained after stopped-flow mixing is shown. The concentrations were: PZP (0.75 μM (molar unit: tetrameric PZP)) and chymotrypsin (1) 0.35; (2) 0.7; (3) 1.0; (4) 1.7; (5) 5.5; (6) 14 and (7) 28 μM. The lines illustrated represent the experimental results with those given by the fitted double exponential equation (Eq. 1) superimposed.

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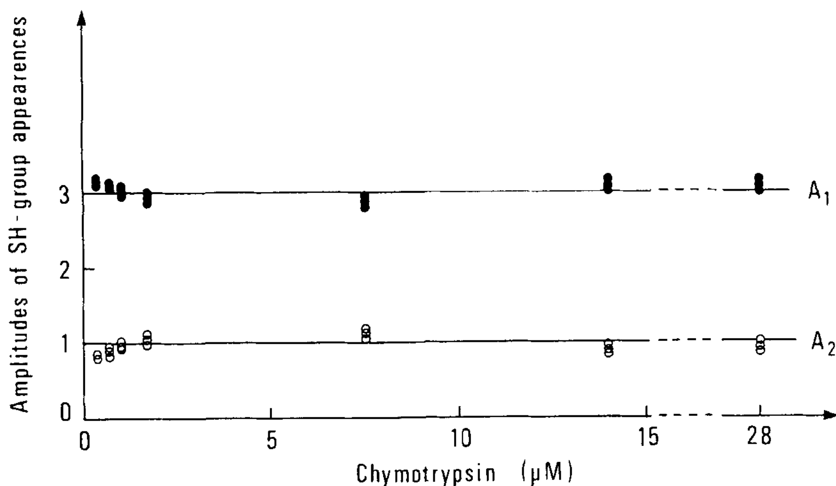

FIGURE 4 Amplitudes according to Eqn. 1 of the faster (A_1) and the slower A_2) sulfhydryl group appearance steps in the reaction of PZP with chymotrypsin shown as a function of the concentration of chymotrypsin. The three different concentrations of PZP used (1.5, 0.75 and 0.38 μM) are all represented. The results were identical within experimental error.

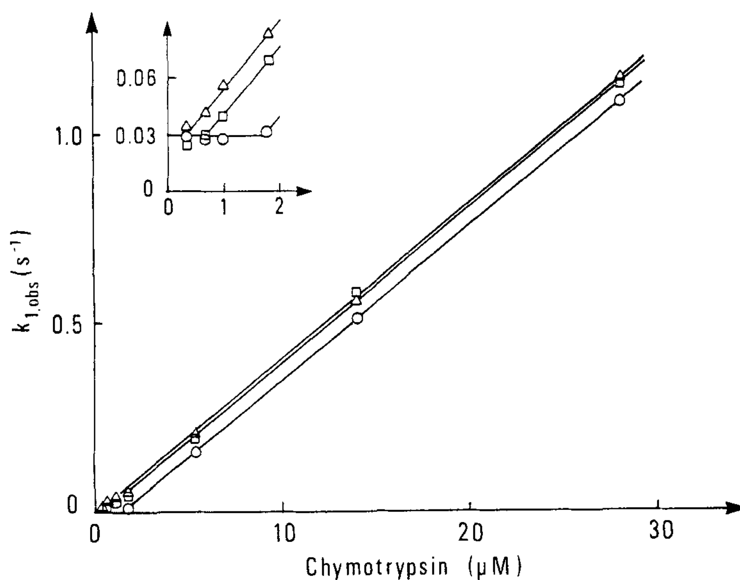

FIGURE 5 The dependence of $k_{1,obs}$ (Eq. 1) on the concentration of chymotrypsin. Insert: The lower concentration range enlarged. The concentrations of PZP were: 0.38 μM (△); 0.75 μM (□) and 1.5 μM (○). The symbol size indicates the confidence limit (c.5%).

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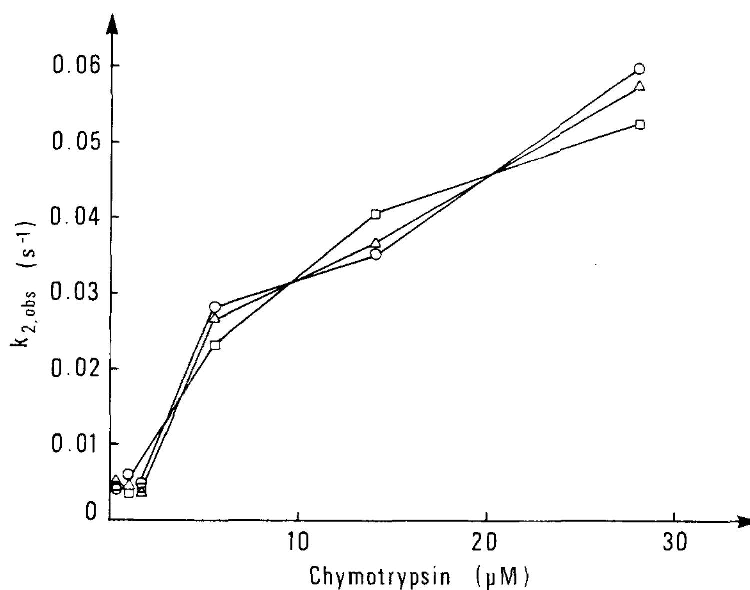

FIGURE 6 The dependence of $k_{2,obs}$ (Eq. 1) on the concentration of chymotrypsin. The concentrations of PZP were: 0.38 μM (△); 0.75 μM (□) and 1.5 μM (○).

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At concentrations of chymotrypsin where PZP is in excess, the rate of formation of three of the four sulfhydryl groups is first order ($k_{1,1} = 0.03\,s^{-1}$) with respect to an equimolar complex of chymotrypsin and PZP-tetramers. When chymotrypsin is in excess, the rate increases and an additional process, which is chymotrypsin-dependent, runs in parallel with the first order decay.

The slow process ($k_{2,obs}$) shows a similar behaviour, but the excess-chymotrypsin dependence is not strictly linear. $k_{2,1} \simeq$ 3-4 $\cdot 10^{-3} s^{-1}$ and $k_{2,2} \simeq 5 \cdot 10^3 M^{-1} s^{-1}$.

Figure 7 shows a number time courses of the decrease of the enzymic activity of chymotrypsin during its reaction with PZP. The curves illustrated were calculated using the rate equation, Eq. (3):

$$v/v_0 = (1 - \exp(-k_{1,obs} t)) \quad (3)$$

which gave results that fitted the experimental data well. v and v_0 are the substrate conversion rates in the presence and absence of PZP, respectively, obtained after the same incubation time.

DISCUSSION

PZP belongs to the family of proteinase-binding α-macroglobulins of which $\alpha_2 M$ is the best characterized member.[1] PZP has the same size of subunits (180 kDa) as $\alpha_2 M$ and their amino acid sequences are 71% identical.[11,12] PZP-subunits contain a bait region quite different from that of $\alpha_2 M$[13] and reacts poorly with trypsin-like proteinases.[3] Interaction of PZP with chymotrypsin involves specific proteolysis of the bait regions, small fluorescence changes and cleavage of internal β-cysteinyl-γ-glytamyl thiol esters of PZP, with resultant covalent binding of the enzyme and appearance of

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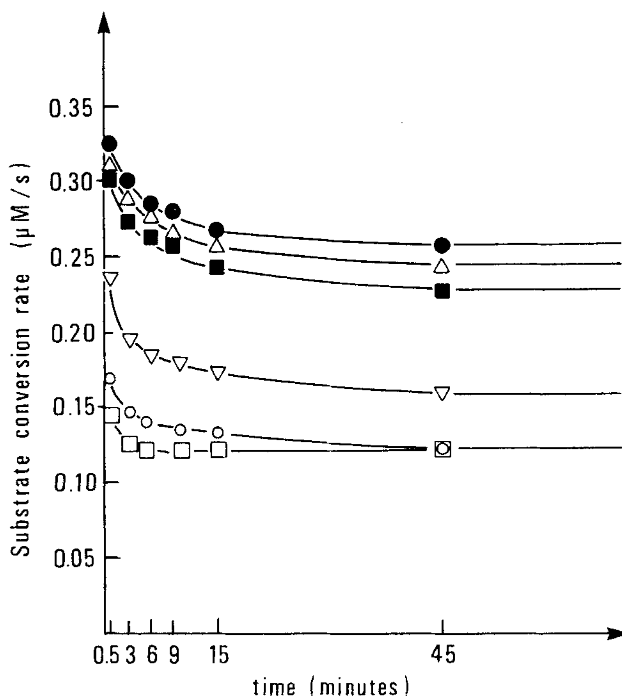

FIGURE 7 Chymotrypsin activity is shown as a function of the time of its incubation with PZP. The activity is given as the initial rate of hydrolysis of Suc-Ala-Ala-Pro-Phe-pNA (μM/s). In the series of experiments illustrated the assay reaction mixtures contained total chymotrypsin (17 nM) and substrate (138 μM), pH 7.6, 25°C. The incubation reaction mixtures contained chymotrypsin (34 nM) and PZP (●) 6 nM; (△) 9 nM; (■) 12.nM; (□) 27 nM; (○) 72.5 nM and (□) 151 nM. The lines show the time courses calculated according to Eq. 3.

sulfhydryl groups. These events are analogous to those known from $\alpha_2 M$-proteinase reactions. One of the major differences between $\alpha_2 M$ and PZP is the stoichiometry of proteinase binding. $\alpha_2 M$ is a tetramer that may bind two molecules of small proteinases, the size of chymotrypsin, but only one molecule of large proteinases, like plasmin.[6,7,14,15] The binding stoichiometry of PZP corresponds with the formation of only equimolar enzyme-PZP(tetramer) complexes and that result is obtained no matter which feature (e.g., binding of ^{125}I-chymotrypsin, cleavage of thiol esters, change of fluorescense, decrease of enzymic activity) of the reaction is investigated.[3,4] The proteinase binding unit of PZP apparently like $\alpha_2 M$[16] is the tetramer, and since native PZP is dimeric the formation of tetrameric PZP is a step required during the reaction with the proteinase. Association of PZP into tetramers occurs only in the presence of enzymes and therefore most probably requires bait region cleavage, it gives rise to small changes of the intrinsic protein fluorescence of PZP-enzyme mixtures, an event which is fast compared to the rates of thiol ester cleavages.[4]

The kinetics of sulfhydryl group appearance in PZP-chymotrypsin has now been investigated in detail. Reaction with DTNB was used to follow the rate of cleavage of the thiol esters of PZP in its reaction with chymotrypsin. In order to determine the validity of the coupled assay procedure, the reaction of DTNB with pre-reacted PZP

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was investigated (Figure 1) and it was found to be sufficiently fast compared to the thiol ester cleavage reactions. The time courses of the cleavage of PZP-thiol esters in reactions with chymotrypsin were found to follow double exponential decays corresponding to a reaction with at least two steps. This behaviour was observed over a rather broad concentration range (Figures 2 and 3). The concentration dependencies of the two discernable steps show that in the faster step three and in the slower step one of the thiol esters react (Figure 4). Further, the observed rate constants of the faster step (Figure 5) depends on the concentration of chymotrypsin when it is in excess (Eq. 2). The same tendency is seen for the slower step (Figure 6), but the results are less clear cut.

When chymotrypsin binds PZP its enzymic activity is changed. With the substrate Suc-Ala-Ala-Pro-Phe-pNA at pH 7.6, 25°C, k_c decreases (0.42-fold) and k_m increases (1.47-fold).[3] The kinetics of this feature of the PZP-chymotrypsin reaction was also investigated (Figure 7). It was found that the decrease of chymotrypsin activity on Suc-Ala-Ala-Pro-Phe-pNA was concomitant with the appearance of the three sulfhydryl groups of the faster step of thiol ester cleavage.

Based on these and previous results we propose a model of the reaction mechanism of the PZP-proteinase binding reaction. The model is outlined in Figure 8. It is a complex, multistep reaction, which involves proteolytic cleavage of PZP-bait regions, association of dimeric-PZP into tetrameric-PZP in the presence of enzyme, cleavage of internal PZP-thiol esters with concomitant covalent binding of the proteinase. Bait region cleavage seems to be prerequisite for the association of PZP-dimers into tetramers, since no tetramers are formed when PZP reacts with methylamine.[3] Fluorescence changes correspond with the formation of equimolar enzyme-PZP(tetramer) intermediates with a rate determining step dependent on enzyme- and PZP-concentrations, not on the square of the PZP-concentrations.[4] That step therefore probably is the initial bait region cleavage step, $k = 5 \cdot 10^5 \mathrm{M}^{-1}\mathrm{s}^{-1}$. If we consider a situation with high levels ($\sim 1\ \mu$M) of PZP as in late pregnancy, the pseudo first order rate constant of the formation of the intermediate, EP_4^* is $0.5\,\mathrm{s}^{-1}$, whereas that of the fast thiol ester cleavage step is only $k_{1,\mathrm{obs}} = 0.03\,\mathrm{s}^{-1}$. The intermediate composed of tetrameric-PZP with intact thiol esters and one associated enzyme molecule builds up rapidly, and its subsequent turnover involves relatively slow internal or external reaction of enzyme with PZP-thiol esters. The covalent binding of the enzyme molecule requires only reaction with one thiol ester, but (on average) three of these react simultaneously with the decrease in enzyme activity to the level characteristic of the final covalently bound enzyme. The reaction of more than one enzymic group with the thiol esters is likely, although some hydrolysis probably also occur. Multicovalently linked enzyme-PZP species are indicated from SDS-PAGE studies of the high molecular weight species noted after reaction of PZP with ^{125}I-chymotrypsin,[3] similar to those seen in reaction mixtures of $\alpha_2 M$ and proteinases.[9,16] The faster thiol ester cleavage step shows a linear concentration dependence on the excess of enzyme (Eq. 2). Therefore it is necessary to include in reaction III a pathway in which internally associated enzyme molecules of the intermediates are exchanged with free enzyme molecules. The enzyme molecule finally being covalently bound is not necessarily the one first associated with the PZP. A dynamic exchange of enzyme molecules accordingly goes on in parallel with the covalent binding reaction, when there is excess of enzyme.

After covalent binding of an enzyme molecule the last thiol ester of the complex is hydrolysed. That process is accelerated in the presence of excess enzyme probably due

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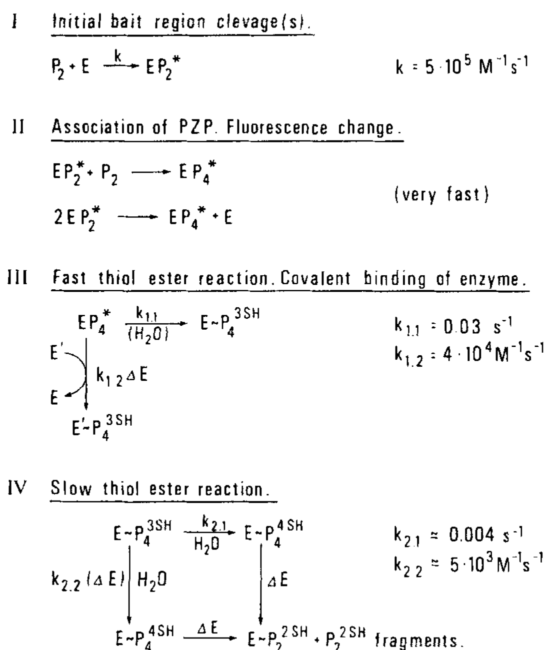

FIGURE 8 Model of the reaction mechanism of the proteinase binding reaction of PZP. I: Bait region cleavage(s) of PZP-dimers, P_2, by the enzyme, E. II: Fast association of enzyme-PZP(dimer)-species, EP_2^* with native PZP, P_2 or with another EP_2^* with dissociation of one of the enzyme molecules. Formation of equimolar enzyme-PZP(tetramer) intermediates, EP_4^*, that show changes of the intrinsic protein fluorescence. Thiol esters intact. III: Reaction of an average of three thiol esters of the intermediate. The associated enzyme molecule or, in excess of enzyme, another enzyme molecule becomes covalently bound, $E \sim P_4^{3SH}$. ΔE is the concentration of enzyme in excess of that necessary for forming a 1 : 1 complex. IV: Hydrolysis of the last thiol ester of the complex and, in the presence of excess of enzyme, unspecific proteolysis of enzyme-PZP(tetramer) complexes and formation of fragments some of which are the size of PZP(dimers) and have enzyme bound.

to non-specific proteolysis of the complex. As has been noted previously[3] excess of chymotrypsin degrades the tetrameric complex and produces fragments, some of which apparently are the size of a PZP-dimer with enzyme bound.

From the discussion presented above it is evident that the proteinase binding mechanism of PZP is analogous to that of tetrameric $\alpha_2 M$. The main difference is that proteolytically cleaved PZP dimers assemble to a tetrameric structure within which covalent proteinase binding takes place, whereas the $\alpha_2 M$ dimers are tightly associated in native $\alpha_2 M$.

The values of the rate constants governing the initial bait region cleavages and the final thiol ester cleavages suggest a considerable lag between these processes. This indicates that initially reversibly bound proteinase molecules may be exchanged and that they are located in different orientations relative to the thiol esters when binding occurs, i.e., binding of proteinase to PZP involves different sets of nucleophiles in individual proteinase molecules. Recently, evidence was presented that this is the case for $\alpha_2 M$.[16]

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