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**MODULATION OF THE PROTEOLYTIC ACTIVITY OF MATRIX  
METALLOPROTEINASE-2 (GELATINASE A) ON FIBRINOGEN**

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**Running Title:** MMP-2 Enzymatic Action on Fibrinogen

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## **SYNOPSIS**

The proteolytic processing of bovine fibrinogen by Gelatinase A (MMP-2), which brings about the formation of a product unable to form fibrin clots, has been studied at 37°C. Catalytic parameters, although showing a somewhat lower catalytic efficiency with respect to thrombin and plasmin, indeed display values perfectly compatible with a meaningful pathophysiological significance of this process. A parallel molecular modelling study predicts a preferential binding of MMP-2 to the  $\beta$ -chain of fibrinogen through its hemopexin-like domain, which has been directly demonstrated by the inhibitory effect in the presence of the exogenous hemopexin-like domain. However, the removal of this domain does not impair the interaction between MMP-2 and fibrinogen, but it certainly alters dramatically the proteolytic mechanism, producing different fragmentation intermediates. The investigation at various pH values between 6.0 and 9.3 displays a proton-linked behaviour, which is relevant for interpreting the influence on the process by environmental conditions occurring in the site of an injury. Furthermore, the action of MMP-2 on peroxynitrite-oxidized fibrinogen has been investigated, a situation possibly occurring under oxidative stress. The chemical alteration of fibrinogen, which has been shown to abolish its clotting activity, brings about only limited modifications of the catalytic parameters without altering the main enzymatic mechanism.

**Keywords: Gelatinase A / Fibrinogen / Fragmentation / Kinetics / Molecular Modeling / pH-Dependence**

**Abbreviations: MMP-2, Gelatinase A; DTPA, diethylenetriamine penta-acetic acid.**

## INTRODUCTION

Matrix metalloproteinases (MMPs) are a class of  $\text{Ca}^{++}$ - and  $\text{Zn}^{++}$ -dependent endopeptidases, displaying an active site characterized by a  $\text{Zn}^{++}$  atom coordinated to three histidyl residues [1]. They show a multi-domain structural organization, usually made by a propeptide domain (which is removed upon enzyme activation), the catalytic domain and a hemopexin-like domain, which are connected by a hinge domain. In addition, a **MMPs subclass, composed of two matrix metalloproteinases MMP-2 and MMP-9 (called gelatinases A and B, respectively) displays a unique collagen binding domain (CBD), called fibronectin-like domain, inserted on the catalytic domain and consisting of three 58-amino acid fibronectin type II-like modules [2].**

These enzymes, which are secreted as inactive zymogens, have the capacity of degrading several proteins of the extracellular matrix, participating in most of the tissue remodelling phenomena [3]. Further, since they are released in the plasma from the polymorphonuclear leukocytes (PMN) [4], it is very likely that they come into close contact with plasma proteins, which can then become physiological substrates for MMPs. As a matter of fact, a growing interest has been addressed toward the role of MMPs in the neovascularization events, which play a key role in several physiological and pathological processes going from tumor dissemination to wound healing. In particular, gelatinase A (MMP-2) is involved in a process termed “angiogenic switch” [5,6], which consists of the induction of a new vasculature by degrading the vascular basement membrane and the extracellular matrix, in order to allow endothelial cells to migrate into the perivascular space. This process can occur at different stages of tumour progression, depending of tumour type and environment. Premalignant lesions as well may induce neovascularization and it has been shown that, during the neovascularization event, the role of several MMPs appears to be that of pericellular fibrinolysins [7], some of them being involved in the degradation of fibrinogen and of cross-linked fibrin necessary to the migration of endothelial cells [8,9]. Therefore, the enzymatic action by MMPs on components of the blood coagulation cascade indeed is relevant both from the physiological and the pathological standpoint.

Fibrinogen is a 340 kDa dimeric glycoprotein, present in the blood, which is composed of six polypeptide chains  $(\alpha\beta\gamma)_2$ , joined by 29 disulphide bridges within the *N*-terminal E domain, forming an elongated 45 nm structure consisting of two outer D-domains connected through a central E-domain by a coiled coil segment. During blood coagulation thrombin converts fibrinogen into fibrin monomers, which associate into staggered, overlapping two-stranded fibrils and these domains contain binding sites which participate in the fibrinogen conversion to fibrin [10]. However, very recent data clearly indicate a correlation between the activity of MMP-2 and MMP-9 and that of fibrinogen in the neovascularization process, since it has been shown that inhibition of the

gelatinase action brings about an accumulation of unprocessed fibrinogen and fibrin, and this is accompanied by a blocking of the neovascularization process [11]. Further, during oxidative stresses connected to the blood vessels damage and their repair a dramatic enhancement of the production of reactive oxidizing (ROS) and nitrosating species (RNS) is observed, leading to vascular injury responsible for the development of atherosclerosis [12]. As a matter of fact, levels of nitrated proteins have been shown to be increased in atherosclerotic lesions [13-16], being predictors of a risk for coronary artery disease [17]. This observation finds an additional support on the very recent observation that post-translational modifications of fibrinogen by reactive nitrogen species, such as peroxynitrite [18], bring about dramatic alterations of the fibrinogen role [19].

Therefore, the elucidation of the mechanism by which the enzymatic activity of MMP-2 on native and oxidized fibrinogen is modulated can be of the utmost importance for a better comprehension of the different interplays between MMPs and coagulation properties and of their physiopathological relevance for the angiogenesis. In the present study we analyze the catalytic efficiency of whole MMP-2 on native and oxidized fibrinogen, comparing it with that of the catalytic domain alone in order to clarify the role of different domains of MMP-2 during fibrinogen degradation. It is interesting to observe that the efficiency of the enzymatic action of MMP-2 on fibrinogen turns out to be somewhat lower than that of thrombin [20] and similar to that displayed by plasmin [21]. Moreover, we accompany this investigation with a molecular modelling of the interaction between MMP-2 and fibrinogen in order to formulate a plausible mechanism based on structural and functional informations. In addition, since pH may vary significantly from neutrality in injured areas and during inflammatory responses, we have carried out an analysis of the pH-dependence of catalytic parameters for the processing of  $\alpha$ - and  $\beta$ -chains of fibrinogen by MMP-2 in order to deepen the information on the modulation of this relevant process by environmental conditions.

## **EXPERIMENTAL**

### ***Materials***

Bovine Fibrinogen (Sigma Chemical Co. St. Louis, MO, USA) was dissolved in water at room temperature to a final concentration of 1 mg/ml. The suspension was centrifuged for 1 hr at 10,000 x g and the supernatant, containing the dissolved fibrinogen, was used. The amount of substrate has been quantified as described by Bradford [22].

Peroxynitrite was synthesized according to the protocol reported by Uppu et al. [23] and stocked at -80°C. Bovine peroxynitrited fibrinogen was prepared by treating native fibrinogen (5 mg/ml) with 40 mM of HONOO, in 50 mM phosphate buffer at pH 7.2 and 0.1 mM DTPA.

MMP-2 proenzyme was either of commercial origin (R&D System, London, UK) or a generous gift of Dr. Chris Overall (Univ. of British Columbia, Canada), and no significant functional difference has been detected between the two preparations after activation. The isolated purified MMP-2 was activated by incubating 0.1 ml of a 0.1  $\mu\text{g/ml}$  progelatinase solution with *p*-aminophenyl mercuric acid (i.e., APMA, Sigma Chemical Co. St. Louis, MO, USA ) at 37°C for 30 minutes and immediately used for experiments. In any case, a control by gelatin zymography showed that over the first 24 hrs no autocatalytic process is taking place under our experimental conditions (data not shown), as already indicated by Nagase et al. [24].

The hemopexin-like domain of MMP-2 was a generous gift of Dr. Chris Overall (Univ. of British Columbia, Canada) and it was prepared as previously reported [25].

Human recombinant MMP-2 catalytic domain (Biomol International, USA) was dissolved in a solution of 50 mM Tris/HCl, 0.1 M NaCl, 10 mM CaCl, pH 7.2.

The quenched fluorogenic substrate MCA-Pro-Leu-Gly-Leu-DPA-Ala-Arg-NH<sub>2</sub> was purchased from Calbiochem (La Jolla, CA, USA).

Plasmin enzyme and the fluorogenic peptide 4-methylumbelliferylguanidinobenzoate (MUGB), an active site titrant of trypsin-like proteases, were of commercial origin (Sigma Chemical Co. St. Louis, MO, USA).

### ***Activity Assay***

The active amount of the enzyme (MMP-2) was determined by gelatin zymography and by the fluorimetric assay, as previously reported [26], following the progressive decrease of hydrolysis of the quenched fluorogenic substrate MCA-Pro-Leu-Gly-Leu-DPA-Ala-Arg-NH<sub>2</sub> upon addition of Batimastat (BB-94), a peptidomimetic inhibitor (kindly provided by British Biotech Pharmaceutical, Cowley, Oxford, UK), which stoichiometrically inhibits MMPs.

### ***Digestion of fibrinogen by MMP-2, plasmin and kinetics***

For substrate fragmentation kinetics, activated whole MMP-2 or plasmin were added to fibrinogen solutions, at a final concentration of 10 pM, while the catalytic domain of MMP-2 was added to fibrinogen solutions at a final concentration of 15-20 pM. The kinetics of MMP-2 has been carried out in 50 mM Tris/HCl, 0.1 M NaCl, 10 mM CaCl<sub>2</sub> at 37°C and at different values of pH, employing different concentrations of bovine fibrinogen (spanning between 5.3  $\mu\text{M}$  and 20  $\mu\text{M}$ ). The same range of substrate concentrations has been employed for peroxy-nitrited fibrinogen, but in this case, as in the case of plasmin, kinetics has been carried out only at pH 7.1. In the case of the experiments in the presence of the hemopexin-like domain of MMP-2, 6  $\mu\text{M}$  fibrinogen has been

equilibrated for 30 min in the presence of 200  $\mu$ M hemopexin-like domain (a concentration required for the almost complete saturation of the binding site, see below), then whole MMP-2 (to a final concentration of 10 pM) was added to start the kinetics.

Kinetics has been carried out keeping the mixtures at 37°C and harvesting small aliquots at different time intervals. Reactions were stopped by the addition of SDS-PAGE loading buffer containing 20 mM EDTA and frozen to – 80°C until used. The aliquots in reducing sample buffer were separated on 10% SDS-PAGE gels, which were stained using 0.5% Coomassie Blue and destained in 10% acetic acid and 40% methanol until substrate bands were clearly visible. The broad spectrum protein markers (Bio Rad, USA) were used as molecular weight standards.

### ***Kinetic Analysis***

Electrophoretic spots, corresponding to different aliquots at different time intervals, have been analysed by a laser densitometer (LKB 2202 UltraScan) and their intensity has been calibrated (in order to obtain concentration values) using standard substrate solutions. It must be pointed out that different preparations of fibrinogen displayed differing ratios of the relative amount of the three chains, as from the intensity of electrophoretic spots on SDS-PAGE under reducing conditions. For  $\alpha$ - and  $\beta$ -chains the substrate disappearance rates were derived at each fibrinogen concentration employed.

The measurement of the initial velocity has been referring to a time period over which less than 10% of the substrate was degraded during the assay; this period never exceeded 10 hrs, which guaranteed the integrity of activated MMP-2, as already reported by Nagase et al. [24]. Therefore, even though we cannot rule out the possibility that at very long time intervals (i.e., > 48 hrs) MMP-2 undergoes a partial autolysis, an investigation outside the purpose of this paper, the catalytic parameters reported in Table I refer to the behaviour observed within the first 10 hrs of reaction. In any case, the analysis was limited to the time interval over which linearity of the rate was observed and it ensured a steady-state condition for the first cleavage step, a prerequisite for the subsequent analysis step. It consisted of the verification for the applicability of the Michaelis-Menten approximation to the first cleavage step, which was based on the observation of an inverse linear correlation between velocity and substrate concentration according to the Lineweaver-Burk equation to obtain the catalytic parameters  $k_{cat}$  and  $K_m$

$$\frac{E_0}{v} = \frac{K_m}{k_{cat}} \cdot \frac{1}{[S]} + \frac{1}{k_{cat}} \quad (\text{Equation 1})$$

where  $E_0$  is the total enzyme concentration,  $v$  is the actual rate (expressed as mol/s),  $K_m$  is the Michaelis-Menten equilibrium constant (expressed as mol),  $k_{cat}$  is the rate-limiting step kinetic constant (expressed as  $s^{-1}$ ) and  $[S]$  is the substrate concentration.

### ***Clotting experiments***

The thrombin-induced clotting of fibrinogen after enzymatic processing by MMP-2 was measured spectrophotometrically, following the increase in absorbance at 350 nm as a function of time, in a Cary 1 dual-beam spectrophotometer, thermostatted at 25°C. Fibrinogen was incubated for 1 min in Tris-HCl buffer 50 mM pH 8.2 and clot formation was triggered by addition of 1.2 units of human thrombin. Clotting curves were analyzed as described elsewhere [27].

### ***Molecular modelling***

Crystal structures isolated from the full length proMMP-2 (PDB #: 1CK7) were used to model the interaction of fibrinogen with different truncated form of gelatinase A (i.e., the autoinhibitory procatalytic domain, the catalytic domain, the catalytic domain lacking the fibronectin-like domain, the fibronectin-like domain alone and the hemopexin-like domain). The dimeric form of fibrinogen (PDB #: 1M1J) is symmetric, so the structure used for the docking simulation was truncated to one monomer plus the *N*-terminal fragments of the second monomer to recreate the interface between the two identical monomers. The docking program employed for the molecular modelling was BiGGER (Biomolecular complex Generation with Global Evaluation and Ranking) [28].

## **RESULTS**

### ***Degradation of fibrinogen and peroxynitrited fibrinogen by MMP-2***

Figure 1A shows the three chains forming the fibrinogen molecule and their enzymatic processing at 37°C and pH 7.1 as a function of incubation time with whole MMP-2. It is interesting to observe that while  $\alpha$ - and  $\beta$ -chains are progressively cleaved by MMP-2,  $\gamma$ -chain turns out to be essentially immune from the cleavage action of MMP-2. This behaviour is significantly different from what reported for the proteolytic action of MMP-8, MMP-12, MMP-13 and MMP-14 on fibrinogen [29], since these MMPs seem to cleave first the  $\alpha$ -chains and only at longer time intervals the cleavage of the  $\beta$ - and  $\gamma$ -chains was taking place. However, it must be pointed out that data in Fig. 1A refer to whole MMP-2, while previous observations [29] were concerning the catalytic domains of MMP-8, MMP-12, MMP-13 and MMP-14, and that an immediate comparison between our kinetics and that reported by others [29] is not possible, since they were using a 1:10 - 1:50 enzyme/fibrinogen molar



ratio while we have been using  $1:10^4$  -  $1:10^5$  enzyme/fibrinogen molar ratio (see Experimental Procedure). Nonetheless, similarly to what reported for the other MMPs [29], we observe the formation of lower molecular weight fragments of the fibrinogen chains, which grow as a function of time of the incubation (Figure 1A). It is important to underline that these fragments do not show any clotting activity upon incubation with thrombin (see Fig. 1B), suggesting that the action of MMP-2 has relevant physiopathological consequences on the coagulation process connected to the impairment of fibrin formation and the promotion of angiogenetic processes. Such a result is not necessarily in contradiction with data reported by Bini et al. [8], who claimed that, unlike for MMP-3, fibrinogen cleaved by MMP-2 showed a slight clot formation after several hours of processing by thrombin. Thus, this statement underlies anyhow a gross alteration of the clotting properties also for the fibrinogen processed by MMP-2, in line with what observed by us for the fibrinogen cleaved by whole MMP-2 (see Fig. 1B).

It is interesting to observe that under the same experimental conditions of pH and temperature plasmin is able to cleave all three chains of fibrinogen (see Fig. 1C) at a different rate (see Table I). In addition, the formation of fragment is observed, which shows a similar molecular weight as that observed in the case of MMP-2, suggesting that the cleavage of the intact chains by plasmin occurs at a site topologically close to that of MMP-2. However, unlike the enzymatic action by MMP-2, in the case of plasmin we also observed at short intermediate times the formation of larger fragments (likely deriving from the cleavage of  $\alpha$ -chains and of  $\beta$ -chains), which then disappear as the smaller fragment builds up (see Fig. 1C), indicating that plasmin could have an additional more efficient cleavage site.

Furthermore, fibrinogen reacted with peroxynitrite (ONOO<sup>-</sup>), thus already unable of any clotting activity, even in the presence of thrombin [18], displays a similar proteolytic processing by whole MMP-2 (Figure 1D), producing a similar fragment as that observed for native fibrinogen, which suggests that the cleavage event(s) occur(s) at a similar site in native and in the peroxynitrite-reacted fibrinogen. It is important to underline that no fragments of smaller molecular weight have been observed, as from gradient electrophoresis observations (**data not shown**), ruling out the possibility of further fragmentation of the fibrinogen chains.

#### ***Degradation of fibrinogen by the catalytic domain of MMP-2***

In order to clarify the role played by the hemopexin-like domain of MMP-2, we have also investigated the enzymatic processing of native fibrinogen by the catalytic domain of MMP-2. Figure 1E reports the time-dependent degradation of the three chains of fibrinogen by the catalytic domain of MMP-2 at pH 7.1 and 37°C. It is immediately obvious that i) the fragmentation occurs

at a much lower rate with respect to the whole MMP-2 (see Figs. 1A and 2), and ii) the proteolytic processing leads to the formation of a new fragment with a molecular weight intermediate between the  $\beta$ - and the  $\gamma$ -chain. This fragment looks similar to that observed during the early stages of the fibrinogen enzymatic processing by plasmin (see Fig. 1C), but it is definitely not observed during the processing of native fibrinogen by whole MMP-2 (see Figs. 1A and 1E). This difference comes out even more evident if we compare the time evolution of the different fragments building up with respect to the disappearance of the intact chains (Figure 2); thus, while in the case of whole MMP-2 the fragment(s) grow(s) up concomitantly with the disappearance of the  $\beta$ -chain (Fig. 2A), in the case of the fragment(s) formed by the catalytic domain of MMP-2 the time evolution of its appearance is correlated to the disappearance of both  $\alpha$ - and  $\beta$ -chains (Fig. 2B). As a whole, these data seem to indicate that cleavage of the  $\alpha$ -chains by whole MMP-2 does not produce an amount of fragments of size large enough to be detected while cleavage of  $\beta$ -chains by whole MMP-2 occurs at a preferential class of sites, with the production of sizeable fragments. Furthermore, the lack of the hemopexin-like domain, when we have only the catalytic domain (see Fig. 1E), brings about cleavage events at sites additional with respect to those involved in the fragmentation by the whole MMP-2. Altogether, these data underly an important role by the hemopexin-like domain in the correct recognition process, as already observed for MMP-8 toward collagen I [30]. It must be underlined that the clotting activity has been abolished also in the case of fibrinogen processed by the catalytic domain of MMP-2 (data not shown), in a fashion closely similar to that observed for the fibrinogen processed by whole MMP-2 (see Fig. 1B). It clearly envisages an important pathophysiological role of MMP-2 in modulating the clotting activity of fibrinogen, independently of its molecular state.

In all cases (i.e., whole MMP-2 and catalytic domain with native and ONOO<sup>-</sup>-treated fibrinogen) the double reciprocal plot of the fibrinogen concentration and the enzymatic activity is linear for both chains of fibrinogen, clearly indicating that the first step occurs by a Michaelis-Menten mechanism (Figure 3). This consideration allows to obtain the catalytic parameters for the cleavage of  $\alpha$ - and  $\beta$ -chains by whole MMP-2 (Fig. 3A) and its catalytic domain (Fig. 3C) of native fibrinogen and by whole MMP-2 of fibrinogen oxidized by peroxynitrite (Fig. 3B). They are reported in Table I, showing that the lower enzymatic efficiency of the catalytic domain alone is completely due to a dramatically reduced kinetic constant for the rate-limiting cleavage step of the substrate.

### *Molecular modelling*

In order to verify whether the functional data described above can find a structural explanation, we have carried out a molecular modelling of the interaction between MMP-2 and fibrinogen. Given that MMP2 is likely to be flexible due to the long linker connecting the hemopexin domain to the catalytic domain, it would not be fruitful to attempt to dock the X-ray structure of the complete enzyme with fibrinogen, as a rigid body. Therefore, for the simulations of the docking between whole MMP2 and fibrinogen we have regenerated a “flexible MMP2”, deleting the 20 amino acidic residues of the linker and forcing the distance between the N-term of the catalytic domain and the C-term of the hemopexin domain not to be longer than 20Å during the docking simulations. This gave us the possibility of comparing the results for the two domains and, using constrained docking, to attempt to simulate the mechanism by which the hemopexin-like domain may bring the catalytic domain into contact with fibrinogen at the correct region.

Figure 4A shows the results for the simulated docking of the catalytic domain (top panel) and the hemopexin-like domain (bottom panel) with fibrinogen. Both panels show 1000 models, with the domain of MMP-2 represented by a coloured sphere placed at its geometric centre in each model. The spheres are coloured according to the electrostatic interaction score, being red for the strongest (most negative) interactions, and green for the weakest one. We chose the electrostatic interaction score as the most representative of the interaction strength because it dominates strongly in the estimates given by BiGGER, with electrostatic energy being approximately 50 times stronger than hydrophobic effects.

These results (Fig. 4A) suggest that the binding of the hemopexin-like domain is more specific, since a large fraction of the models place the hemopexin-like domain near the C terminal region of fibrinogen (to the left on Figs. 4A and 4B), whereas the catalytic domain models are more evenly spread throughout the structure of fibrinogen. Not apparent in the figure, but also important, the top electrostatic interaction score given by BiGGER is 50% higher for the hemopexin-like domain than it is for the catalytic domain (-152 kCal/mol vs. -110 kCal/mol), suggesting that the interaction of the hemopexin-like domain is not only more specific but also stronger.

The results shown in Figures 4A and 4B are for the docking of the active form of the catalytic domain, but we also used the autoinhibitory procatalytic form of the catalytic domain, the catalytic domain lacking the fibronectin-like motif, and the fibronectin-like motif alone (results not shown). These simulations gave the expected results, producing neither a pattern of specific interactions nor interactions stronger than those for the active form of the catalytic domain of MMP-2.

A closer examination of highest scoring models for the hemopexin-like domain docking indicates preferential binding sites which are grouped near the globular regions of the  $\beta$ -chains of the fibrinogen molecule. We selected the five highest ranking models from this simulation (according to the electrostatic score), and used the respective five complexes of fibrinogen with the hemopexin-like domain as targets for docking the catalytic domain of MMP-2. In addition, we imposed a constraint of 20 Å for the distance between the *N*-terminal of the hemopexin domain and the *C*-terminal of the catalytic domain, which corresponds to the length of the linker segment we deleted when separating these two domains from the X-ray structure of the complete enzyme [31] (see the first section describing MMP-2). Figure 4B (bottom panel) shows the models from these five docking simulations in which the catalytic Zn<sup>++</sup> atom of the catalytic domain is within 5Å of the backbone of fibrinogen. The justification for this selection is the assumption that the hemopexin-like domain is responsible for the specific and stable binding to the fibrinogen, placing the catalytic domain near the regions where the backbone of fibrinogen is accessible to the catalytic centre. Figure 4B compares these constrained docking results with a similar selection (catalytic Zn<sup>++</sup> within 5Å of fibrinogen backbone) of the models obtained by docking the catalytic domain alone to the fibrinogen with no constraints.

These docking simulation results are in agreement with the experimental results of the digestion of fibrinogen by MMP-2, which show that the hemopexin-like domain greatly increases the catalytic rate of the catalytic domain, and suggest that the catalytic domain alone cleaves fibrinogen with less specificity than the complete enzyme does, leading to the formation of additional fragments (see Fig. 1E). Comparing the unconstrained and constrained docking simulations for the catalytic domain (Fig. 4B) we can see a similar pattern of models where the catalytic Zn<sup>++</sup> can interact with the fibrinogen backbone, but with lower specificity in the case of the unconstrained docking (top panel), which shows a more disperse distribution of the catalytic domain. More significantly, the docking simulations indicate cleavage sites only on the  $\alpha$  and  $\beta$  chains, represented respectively in yellow and green (Fig. 4B), and none on the  $\gamma$ -chain, again in agreement with the experimental data on the digestion kinetics.

An analogous analysis has not been possible for the  $\alpha$ -chain, since most of this chain is not visible from crystallographic available data due to a widespread disordered structural arrangement of this chain. As a matter of fact, docking simulations focussed on the available portion of  $\alpha$ -chain didn't show any preferential interaction with whole MMP-2.

### ***Effect of the hemopexin-like domain on the digestion of fibrinogen by whole MMP-2***

In order to test the reliability of predictions from molecular modelling on the crucial role played by the hemopexin-like domain in the interaction between whole MMP-2 and the  $\beta$ -chain (and probably the  $\alpha$ -chain) of fibrinogen (see Fig. 4), we have carried out a fragmentation kinetics by whole MMP-2 of fibrinogen incubated at 37°C with 200  $\mu$ M hemopexin-like domain. Thus, if the hypothesis suggested by the molecular modelling is correct, at such a concentration the hemopexin-like domain should almost fully occupy the binding site, as from the  $K_m$  value (see Table I), severely impairing the fragmentation of fibrinogen by whole MMP-2. Figure 5 reports the outcome of this experiment carried out in parallel with the fragmentation of fibrinogen alone, clearly showing that the presence of 200  $\mu$ M hemopexin-like domain greatly inhibits the fragmentation of  $\alpha$ - and  $\beta$ -chains of fibrinogen. This is a very important result, since it

- i) directly demonstrates the validity of the hypothesis formulated on the basis of molecular modelling, allowing to propose the interaction of the hemopexin-like domain with the  $\beta$ -chain of fibrinogen as an actual mechanism and not simply as a working hypothesis;
- ii) rules out the possibility that other contaminating MMPs contribute significantly to the observed phenomenon, since the hemopexin-like domain can only interfere with intact MMP-2 and its reaction with fibrinogen is shown to essentially abolish the proteolytic cleavage.

### ***The pH-dependence of catalytic parameters for MMP-2 on fibrinogen***

The pH-dependence of catalytic parameters for MMP-2 on the  $\alpha$ - and  $\beta$ -chains of native fibrinogen has been characterized and the values of  $pK_{aU}$  (corresponding to values in the free enzyme) and  $pK_{aL}$  (corresponding to values in the substrate-bound enzyme) have been determined on the basis of the pH-dependence of different catalytic parameters according to the following equations [26]

$$\left(\frac{k_{cat}}{K_m}\right)_{obs} = \frac{\sum_{i=0}^{i=n} \frac{k_{cat}}{K_{mi}} \prod_{r=0}^{r=i} K_{aUr} \cdot [H^+]}{\sum_{i=0}^{i=n} \prod_{r=0}^{r=i} K_{aUr} \cdot [H^+]} \quad (\text{Eq. 2a})$$

$${}_{obs}k_{cat} = \frac{\sum_{i=0}^{i=n} k_{cati} \prod_{r=0}^{r=i} K_{aLr} \cdot [H^+]}{\sum_{i=0}^{i=n} \prod_{r=0}^{r=i} K_{aLr} \cdot [H^+]} \quad (\text{Eq. 2b})$$

$${}^{obs}K_m = K_{m0} \cdot \frac{\sum_{i=0}^{i=n} \prod_{r=0}^{r=i} K_{aUr} \cdot [H^+]}{\sum_{i=0}^{i=n} \prod_{r=0}^{r=i} K_{aLr} \cdot [H^+]} \quad (\text{Eq. 2c})$$

where  ${}^{obs}(k_{cat}/K_m)$ ,  ${}^{obs}k_{cat}$  and  ${}^{obs}K_m$  are the values of these catalytic parameters at a given pH,  $i$  ( $= 0, 1, \dots, n$ ) are the different protonation states (with  $(k_{cat}/K_m)_0$ ,  $k_{cat0}$  and  $K_{m0}$  the values of the catalytic parameters in the unprotonated form),  $K_{aUi}$  ( $= 10^{pK_{aUi}}$ ) and  $K_{aLi}$  ( $= 10^{pK_{aLi}}$ ) are the  $i$ th proton binding constants for the free reactants and the enzyme-substrate complex, respectively, and  $[H^+]$  is the proton concentration. Experimental data have been fitted with Eqs. (2a, 2b and 2c), employing  $n = 3$  and this proton-linked modulation is described in Figure 6 with parameters reported in Table II.

Data in Table II make it very clear that the pH-dependence profile of the overall enzymatic activity (i.e.,  $k_{cat}/K_m$ ) is closely similar for the two chains and quite complex (see Fig. 6A), requiring the involvement of (at least) three protonating groups. On the other hand, the pH-dependence of the rate-limiting step (i.e.,  $k_{cat}$ ) shows a somewhat simpler bell-shaped profile and it appears to be slightly displaced to more alkaline pH values in the case of the  $\beta$ -chain (see Fig. 6B). As a consequence, a similar displacement is observed for  $K_m$  (see Fig. 6C), which is affected only by those groups whose  $pK_a$  values change upon substrate binding (see Table II).

It is important to remark that resulting  $pK_a$  values are different from those reported previously for MMP-2 against synthetic substrates [26]; this is quite obvious, since different residues are involved in the two cases, as implied by the fact that MMP-2 interacts through the hemopexin-like domain with fibrinogen, whereas the binding of synthetic substrates only involves residues in the immediate vicinity of the catalytic site. Obviously, we cannot rule out the possibility that some of these  $pK_a$  values refer also to residue(s) of fibrinogen, which change their value upon interaction with MMP-2.

From an overall view of the pH-dependence of these three catalytic parameters it comes out that the decrease of  $k_{cat}/K_m$  between pH 9.5 and pH 8.5 (see Fig. 6A) is due to a marked increase of  $K_m$  (that is a decrease of substrate affinity) over the same pH range (see Fig. 6C), which is only modestly compensated by a small increase of  $k_{cat}$  (see Fig. 6B). On the other hand, the enhancement of  $k_{cat}/K_m$  between pH 8.5 and 7.0 (see Fig. 6A) is mostly due to the increase of  $k_{cat}$ , which attains the maximum value at pH 7.2 for the  $\alpha$ -chain and at pH 7.5 for the  $\beta$ -chain (see Fig. 6B). Over the same range,  $K_m$  is giving only a minor contribution, since it attains the minimum affinity value at pH 7.4 for the  $\alpha$ -chain and at pH 7.8 for the  $\beta$ -chain (see Fig. 6C). The decrease of  $k_{cat}/K_m$  at pH  $< 7.0$  (see Fig. 6A) is again induced by the decrease of the rate-limiting step  $k_{cat}$  (see Fig. 6B), which

overwhelms the increasing affinity of MMP-2 for the substrate (as indicated by the decrease of  $K_m$ , see Fig. 6C). Therefore, we can conclude that the proteolytic activity of MMP-2 towards the two chains of fibrinogen is dominated at alkaline pH values by the substrate affinity, while as the pH is lowered the catalytic efficiency becomes dominated by the velocity of the rate-limiting step.

## DISCUSSION

The role of MMP-2 in the neovascularization process is very well established and so is its capability to enzymatically process fibrinogen and cross-linked fibrin [8]. A very important aspect of the enzymatic action of MMP-2 on fibrinogen is the observation that the cleavage brings about an impairment to the formation of fibrin clots upon exposure to the action of thrombin (see Fig. 1B). It clearly means that this process induces a gross alteration of the coagulation process with important pathological consequences. Therefore, since MMP-2 and fibrinogen are coming into close contact in the bloodstream, a kinetic analysis of the proteolytic processing by whole MMP-2 of native fibrinogen and of peroxynitrite-treated fibrinogen (a condition present under oxidative stress and which is known to impair the formation of clots [18]) is of the utmost importance for the comprehension of such a relevant event. This is clearly demonstrated by the fact that the catalytic efficiency of MMP-2 on fibrinogen (as indicated by  $k_{cat}/K_m$ ) is somewhat lower than that of thrombin [20] and similar to that displayed by plasmin [21]. In addition, in order to uncover the determinants of the enzyme-substrate recognition process, the enzymatic inactivation of native fibrinogen by the catalytic domain of MMP-2 and by intact MMP-2 in the presence of a saturating amount of hemopexin-like domain has been investigated, allowing to establish unequivocally the role of the hemopexin-like domain in this relevant process. Furthermore, the pH-dependence of catalytic parameters for the processing of both fibrinogen chains by whole MMP-2 allows to clarify the substrate binding mechanism and the modulation of enzymatic activity by environmental conditions.

Table I reports the catalytic parameters for this process at pH 7.1 and 37°C, which indicate that the overall enzymatic activity, as indicated by values of  $k_{cat}/K_m$ , of intact MMP-2 is faster on the  $\alpha$ -chain as compared to the  $\beta$ -chain of native fibrinogen (mostly referable to a faster  $k_{cat}$ , see Table I), while it is closely similar for the two chains in the ONOO<sup>-</sup>-oxidized fibrinogen. Further, the removal of the hemopexin-like domain brings about a dramatic 20-fold reduction in the enzymatic activity with respect to the intact MMP-2, though keeping the about 3-fold preferential action for the  $\alpha$ -chain (mostly referable a difference for  $K_m$ , see Table I), suggesting a difference for the substrate recognition process according to whether MMP-2 has the hemopexin-like domain or not.

This is in accord with a variation in the binding mode between the whole MMP-2 and the catalytic domain. Thus, in the first case binding is driven by the hemopexin-like domain (which has a similar affinity for the two chains, as suggested by the similar  $K_m$  of intact MMP-2, see Table I) while in the second case the process is regulated by the interaction of the catalytic domain (which interacts differently with the cleavage site of the two chains, as also suggested by the different  $k_{cat}$  in the whole MMP-2, see Table I). This result, which may find a support on data on collagen by others [32,33], would suggest that MMP-2 interacts with fibrinogen by first binding through the hemopexin domain, which then acts as an anchor to force the catalytic domain into contact with the substrate. The occurrence of this interaction, which would be also in accord with previous data indicating that binding of the hemopexin-like domain is driven by electrostatic forces [34], indeed is demonstrated unequivocally by the experiment, shown in Fig. 5, which puts in evidence as in the presence of the hemopexin-like domain the degradation of fibrinogen by whole MMP-2 is severely impaired. As a whole, at pH 7.1 it emerges a picture in which whole MMP-2 binds with equal probability  $\alpha$  and  $\beta$  chains of native fibrinogen (as from the closely similar values of  $K_m$ , see Table I) by means of the hemopexin-like domain (see Fig. 4). Moreover, data reported in Table I indicate that MMP-2 cleaves the two chains at a different rate, mostly because the catalytic domain appears to preferentially process the  $\alpha$ -chain, which is likely more exposed.

It is interesting to observe that comparing the behaviour of intact MMP-2 and plasmin shows a drastic difference in that plasmin is able to enzymatically process also the  $\beta$ -chain of fibrinogen (see Fig. 1C), while intact MMP-2 doesn't. Furthermore, observing the catalytic parameters reported in Table I, it comes out that the catalytic efficiency (as expressed by  $k_{cat}/K_m$ ) of intact MMP-2 is closely similar to that of plasmin on the  $\alpha$ -chain of fibrinogen, as a result of a counterbalancing between the velocity of the rate-limiting step (as expressed by  $k_{cat}$ ), which is faster for intact MMP-2 and the substrate affinity (as expressed by  $K_m$ ), which is higher for plasmin. On the other hand, for the  $\beta$ -chain of fibrinogen plasmin shows a fourfold better activity with respect to intact MMP-2, which is almost fully attributable to a faster  $k_{cat}$  (see Table I). As a whole, it looks like that the enzymatic action of plasmin and MMP-2 are fairly similar with the exception of the fact that plasmin is able to cleave the  $\beta$ -chain, likely reflecting the smaller size of the enzyme and its higher flexibility.

Interpretation of data obtained for the catalytic domain of MMP-2 on native fibrinogen appears somewhat puzzling, as compared with those of intact MMP-2. Thus, the removal of the hemopexin-like domain seems to bring about a dramatic decrease by two orders of magnitude for the rate constant of the rate-limiting step (i.e.,  $k_{cat}$ ), partially compensated by an increased affinity by about one order of magnitude for both chains (as from the decrease of  $K_m$ , see Table I). At first sight, this



result seems to contradict the relevance of the role played by the hemopexin-like domain in the substrate recognition, since, this being the case, we would have expected a dramatic decrease of affinity, and thus an increase of  $K_m$ , upon removal of the hemopexin-like domain. Actually, these data can be somehow reconciled with the picture given above for the whole MMP-2, suggesting a much more complex role of the hemopexin-like domain. Thus, the drastically different value of  $k_{cat}$  underlies a “correct” substrate recognition (such that MMP-2 is able to cleave fibrinogen at the correct place) only in the presence of the hemopexin-like domain, suggesting an “unproductive” ES complex in its absence, as also observed for collagen I [25,30,31,35]. Therefore, the decrease of  $K_m$  (see Table I) for the catalytic domain may be attributed to the appearance, consequent to the removal of the hemopexin-like domain, of a new interaction site between MMP-2 and fibrinogen, with the consequent formation of new fragments (see Figs. 1E and 2B) at a much less efficient rate.

A different effect must come into play in the cleavage mechanism of ONOO<sup>-</sup>-oxidized fibrinogen by whole MMP-2. In this case, it appears as if the ONOO<sup>-</sup>-induced oxidation of fibrinogen brings about a structural alteration of the molecule, which renders it more easily recognized by MMP-2 (as from the lower value of  $K_m$ , see Table I), but more resistant to the cleavage action (as from the lower value of  $k_{cat}$ , see Table I). The net result is a similar overall enzymatic action by MMP-2 with respect to native fibrinogen (as from the similar value of  $k_{cat}/K_m$ , see Table I, and the same type of fragment produced by the cleavage action, see Fig. 1). This conformational change occurring in ONOO<sup>-</sup>-oxidized fibrinogen is also likely responsible for the disappearance of catalytic heterogeneity of the two chains. It renders much stronger the interaction of MMP-2 with both chains of fibrinogen (this feature being particularly evident for the  $\beta$  chain), but it also leads to a drastically reduced susceptibility of both chains to the proteolytic cleavage (the effect being especially pronounced for the  $\beta$  chain, see Table I).

An interesting aspect of catalytic parameters for the activity of intact MMP-2 on native fibrinogen is the close similarity of  $K_m$  for both  $\alpha$ - and  $\beta$ -chains (see Table I). The whole MMP-2 has the same affinity for  $\alpha$ - and  $\beta$ -chains, envisaging the possibility that there is only one binding site for MMP-2 on fibrinogen. This being true, the binding site could be located near the globular D domain of  $\beta$ -chain, as suggested above by the molecular modelling (see Fig. 4) and by the experiment in the presence of the hemopexin-like domain (see Fig. 5), and the same bound molecule could alternatively cleave either the  $\alpha$ - and/or  $\beta$ -chains.

In order to discriminate between this possibility of a single binding site and that in which fibrinogen has two different binding sites for MMP-2 (one on the  $\alpha$ -chain and one on the  $\beta$ -chain), we have investigated the pH-dependence of catalytic parameters for MMP-2 on the  $\alpha$ - and  $\beta$ -chains of native fibrinogen. Thus, following this approach the occurrence of a single binding site in

fibrinogen for MMP-2 demands that the pH-dependence for  $K_m$  is the same in the case of  $\alpha$ - and  $\beta$ -chain. This possibility is ruled out by the different pH-dependence of  $K_m$  for the two chains (see Fig. 6C), unequivocally demonstrating that in fibrinogen there are two independent binding sites for MMP-2 (one at the  $\alpha$ -chain and one at the  $\beta$ -chain).

A closer look at parameters reported in Table II shows that all three  $pK_{aU}$  values are the same for both fibrinogen chains, indicating that the same groups of MMP-2 are involved in the proton-linked modulation of catalytic properties toward the two chains. This is also an indirect indication that two different MMP2 molecules interact with the two chains, even though the interaction mode must differ for the two chains, as from the different  $pK_a$  shifts (resulting in different  $pK_{aL}$  values, see Table II).

The identification of groups involved in this proton-linked modulation of the enzymatic activity of MMP-2 towards fibrinogen is quite difficult, since an accurate identification would require an extensive study employing site-directed mutants. On the other hand, we cannot rule out the possibility that also some of  $pK_a$  values refer to residue(s) of fibrinogen, which change their values upon interaction with MMP-2. However, some tentative attribution can be undertaken based on the  $pK_a$  values and on their shift, taking into consideration the fact that not all three groups must necessarily be in the close proximity of the active site.

In this respect, the residue responsible for  $pK_{a1}$ , which displays a fairly high  $pK_{aU}$  value (possibly referable to a tyrosine) before the enzyme:fibrinogen interaction, undergoes a drastic shift with a proton release upon substrate binding, much more pronounced for the interaction of MMP-2 with the  $\beta$ -chain (see Table II). This behaviour suggests that in the ES complex the environment is very polar and positively charged, with a consequent marked enhancement of its acidic character, which might render very difficult its protonation down to fairly low pH values.

On the other hand, the residue responsible for  $pK_{a2}$ , which shows a quite low value for  $pK_{aU}$  before the interaction (possibly referable to a histidine), changes very little upon interaction of MMP-2 with the  $\alpha$ -chain, but it is upshifted to a much larger extent when the enzyme interacts with the  $\beta$ -chain (see Table II). The varying behaviour for the two chains suggests that this residue might be located in a region of MMP-2 which interacts with two drastically different regions of the two chains. Thus, unlike what observed in the case of the residue responsible for  $pK_{a1}$  (see above), this residue weakens its acidic character taking up a proton upon interaction with the  $\beta$ -chain, while it is very marginally affected by the interaction with the  $\alpha$ -chain.

In the case of the residue responsible for the  $pK_{a3}$ , it displays a very marginal  $pK_a$  increase upon substrate binding (to a similar extent for the two chains, see Table II), suggesting only a modest change in the environment upon substrate binding for both chains.

In conclusion, from this study several novel aspects of this relevant process emerge, such that i) different domains of MMP-2 contribute in a synergistic way to the proteolytic processing of fibrinogen, ii) this proteolytic cleavage brings about the formation of an inactive form of fibrinogen, thus grossly affecting the coagulation process, iii) MMP-2 binds with the hemopexin-like domain, **enzymatically processing** both  $\alpha$ - and  $\beta$ -chains, **while there is no evidence for the cleavage of the  $\gamma$ -chain**, iv) the ONOO<sup>-</sup> oxidized fibrinogen undergoes a conformational change, which affects the enzymatic mechanism by which MMP-2 processes fibrinogen, and v) the pH-dependence of catalytic parameters for MMP-2 on fibrinogen required at least three protonating groups whose pK<sub>a</sub> values change upon substrate binding.

A final comment must underline that inactivation of fibrinogen by both whole MMP-2 and the catalytic domain of MMP-2 is of great pathophysiological relevance in view of values of the catalytic parameters, not dramatically different from those observed for thrombin and plasmin [20,21] and for the important role played by MMP-2 in promoting inflammation and in favouring angiogenesis [7-9]. These data give also a functional support to the crucial role played by MMP-2 in inducing vascular and circulatory alterations under chronic inflammatory conditions, such as those occurring in atherosclerosis and diabetes [36,37].

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**TABLE I**

Catalytic parameters for the enzymatic processing at 37°C and pH 7.1 of native fibrinogen by the whole MMP-2, the catalytic domain of MMP-2 and plasmin and of ONOO<sup>-</sup>-treated fibrinogen by whole MMP-2.

	$k_{\text{cat}}/K_{\text{m}}$ ( $\text{M}^{-1} \text{s}^{-1}$ )	$k_{\text{cat}}$ ( $\text{s}^{-1}$ )	$K_{\text{m}}$ (M)
<b><math>\alpha</math>-chain</b>			
<i>Native Fibrinogen</i>			
Whole MMP-2	$2.29(\pm 0.23) \times 10^5$	$36.4(\pm 4.2)$	$1.59(\pm 0.18) \times 10^{-4}$
Catalytic Domain MMP-2	$1.09(\pm 0.12) \times 10^4$	$0.19(\pm 0.02)$	$1.72(\pm 0.19) \times 10^{-5}$
Plasmin	$2.04(\pm 0.21) \times 10^5$	$7.7(\pm 0.9)$	$3.77(\pm 0.41) \times 10^{-5}$
<i>Oxidized Fibrinogen</i>			
Whole MMP-2	$1.05(\pm 0.11) \times 10^5$	$3.1(\pm 0.4)$	$2.93(\pm 0.35) \times 10^{-5}$
<b><math>\beta</math>-chain</b>			
<i>Native Fibrinogen</i>			
Whole MMP-2	$6.61(\pm 0.83) \times 10^4$	$12.5(\pm 1.4)$	$1.89(\pm 0.20) \times 10^{-4}$
Catalytic Domain MMP-2	$3.59(\pm 0.42) \times 10^3$	$0.12(\pm 0.02)$	$3.27(\pm 0.41) \times 10^{-5}$
Plasmin	$2.47(\pm 0.34) \times 10^5$	$27.8(\pm 2.9)$	$1.12(\pm 0.15) \times 10^{-4}$
<i>Oxidized Fibrinogen</i>			
Whole MMP-2	$1.34(\pm 0.15) \times 10^5$	$1.06(\pm 0.12)$	$7.93(\pm 0.88) \times 10^{-6}$
<b><math>\gamma</math>-chain</b>			
<i>Native Fibrinogen</i>			
Plasmin	$4.96(\pm 0.6) \times 10^4$	$2.35(\pm 0.36)$	$4.74(\pm 0.57) \times 10^{-5}$

**TABLE II**

Values of  $pK_{aU}$  and  $pK_{aL}$  for the pH dependence of enzymatic processing of  $\alpha$ - and  $\beta$ -chains of fibrinogen by whole MMP-2 at 37°C.

	<b><math>\alpha</math> chain</b>	<b><math>\beta</math> chain</b>
<b><math>pK_{aU1}</math></b>	<b>10.04±0.15</b>	<b>10.04±0.17</b>
<b><math>pK_{aU2}</math></b>	<b>4.07±0.16</b>	<b>4.07±0.15</b>
<b><math>pK_{aU3}</math></b>	<b>9.97±0.17</b>	<b>9.97±0.18</b>
<b><math>pK_{aL1}</math></b>	<b>7.86±0.16</b>	<b>4.86±0.17</b>
<b><math>pK_{aL2}</math></b>	<b>4.52±0.16</b>	<b>7.72±0.15</b>
<b><math>pK_{aL3}</math></b>	<b>10.05±0.17</b>	<b>10.32±0.17</b>



## LEGEND OF FIGURES

**Figure 1:** (*panel A*) SDS-PAGE electrophoresis of the processing of bovine fibrinogen by whole MMP-2 as a function of time (as indicated) at 37°C and pH 7.1.  $\alpha$ - (66.5 kDa),  $\beta$ - (52 kDa) and  $\gamma$ - (47 kDa) chains are indicated as well as the 40 kDa fragment; (*panel B*) Clotting induced by human thrombin on bovine fibrinogen (*curves a-c*) and on bovine fibrinogen after degradation by whole MMP-2 (*curves d-g*). Fibrinogen was incubated at 37 °C in Tris-HCl buffer 50 mM pH 8.2 and the clotting was followed at 350 nm after addition of 1.2 units of human thrombin. *Curves a-c*: 0.24, 0.16 and 0.08 mg/ml of bovine fibrinogen. *Curves d-g*: 0.08, 0.16, 0.24 and 0.32 mg/ml of fibrinogen degraded by whole MMP-2; (*panel C*) SDS-PAGE electrophoresis of the processing of bovine fibrinogen by plasmin as a function of time (as indicated) at 37°C and pH 7.1  $\alpha$ - (66.5 kDa),  $\beta$ - (52 kDa) and  $\gamma$ - (47 kDa) chains are indicated as well as the 40 kDa fragment. (*panel D*) SDS-PAGE electrophoresis of the processing of ONOO<sup>-</sup>-oxidized bovine fibrinogen by MMP-2 as a function of time (as indicated) at 37°C and pH 7.1.  $\alpha$ - (66.5 kDa),  $\beta$ - (52 kDa) and  $\gamma$ - (47 kDa) chains are indicated as well as the 40 kDa fragment; (*panel E*) SDS-PAGE electrophoresis of the processing of bovine fibrinogen by the catalytic domain of MMP-2 as a function of time (as indicated) at 37°C and pH 7.1.  $\alpha$ - (66.5 kDa),  $\beta$ - (52 kDa) and  $\gamma$ - (47 kDa) chains are indicated as well as the 50 kDa and the 40 kDa fragment. For further details, see text.

**Figure 2:** (*panel A*) Temporal evolution of the  $\alpha$ - ( $\square$ ),  $\beta$ - ( $\blacktriangledown$ ) chains degradation and of the 40 kDa fragment formation ( $\Delta$ ) during the processing of bovine fibrinogen by whole MMP-2. Amounts of each species are expressed in terms of number of moles. (*panel B*) Temporal evolution of the  $\alpha$ - ( $\square$ ),  $\beta$ - ( $\blacktriangledown$ ) chains degradation and of the 50 kDa (o) and 40 kDa ( $\Delta$ ) fragment formation during the processing of bovine fibrinogen by the catalytic domain of MMP-2. Amounts of each species are expressed in terms of number of moles. T = 37°C and pH = 7.1. For further details, see text.

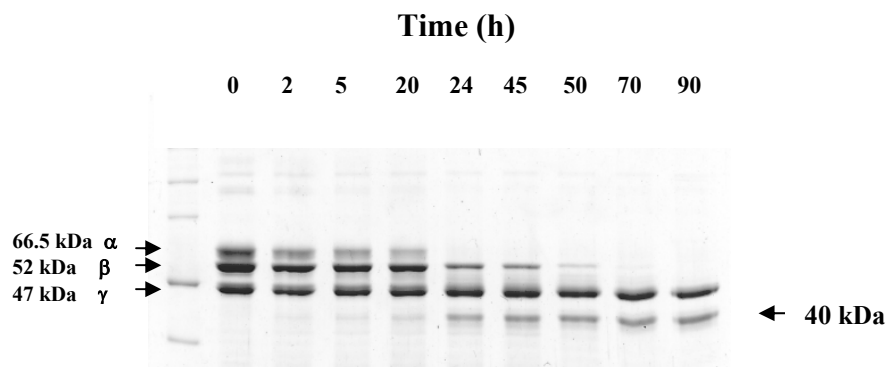
**Figure 3:** Lineweaver-Burk plots of the enzymatic processing by whole MMP-2 of  $\alpha$ - ( $\Delta$ ) and  $\beta$ - ( $\square$ ) chains of native fibrinogen (*panel A*), ONOO<sup>-</sup>-oxidized fibrinogen (*panel B*) and of native fibrinogen by the catalytic domain of MMP-2 (*panel C*) at 37°C and pH 7.1. Continuous lines have been obtained by non-linear least-squares fitting of data according to Eq. (1) and parameters are reported in Table I. For further details, see text.

**Figure 4:** (*Panel A*) Comparison of 1000 models for the docking of the catalytic domain (top panel) and the hemopexin-like domain (bottom panel) with fibrinogen. The fibrinogen monomer is shown

at the centre of each panel (with  $\alpha$ -,  $\beta$ - and  $\gamma$ -chain identified by orange, green and blue colour, respectively), and each MMP-2 domain is represented by coloured spheres placed at their geometric centres. The models were selected and coloured according to the electrostatic interaction score, with red indicating stronger interactions and green weaker interactions. For the results reported in panel A no constraints were used, as each domain was docked independently to compare the specificity of their interactions. (*Panel B*) Comparison of the regions of fibrinogen found to be accessible to the catalytic domain. Spheres represent the position of the catalytic Zn. The top panel shows the models for the unconstrained docking of the catalytic domain; the bottom panel shows the models for the constrained docking of the catalytic domain with five complexes of the hemopexin-like domain and fibrinogen. Numbers indicate potential cleavage sites on the  $\alpha$ - and  $\beta$ -chains of fibrinogen, as suggested by the docking of the Complex between the catalytic domain and the hemopexin-like domain. For further details, see text.

**Figure 5:** SDS-PAGE electrophoresis of fibrinogen degradation by whole MMP-2 after 20 hrs at 37°C in the absence (*lane 2*) and in the presence of 200  $\mu$ M hemopexin-like domain (*lane 4*). *Lane 3* shows the intact fibrinogen. *Lane 1* shows the low MW markers and *lane 5* shows the broad MW markers. Chains of fibrinogen and the hemopexin-like domain are indicated by arrows For further details, see text.

**Figure 6:** pH dependence of  $k_{\text{cat}}/K_m$  (*panel A*),  $k_{\text{cat}}$  (*panel B*) and  $K_m$  (*panel C*) for the processing of  $\alpha$ - (o) and  $\beta$ - (x) chains of bovine fibrinogen by whole MMP-2 at 37°C. Continuous lines correspond to non-linear least-squares fitting of data according to Eqs. (2a), (2b) and (2c), and values of  $\text{pK}_{\text{aU}}$  and  $\text{pK}_{\text{aL}}$  for the two chains are reported in Table II. For further details, see text.



**Figure 1A**

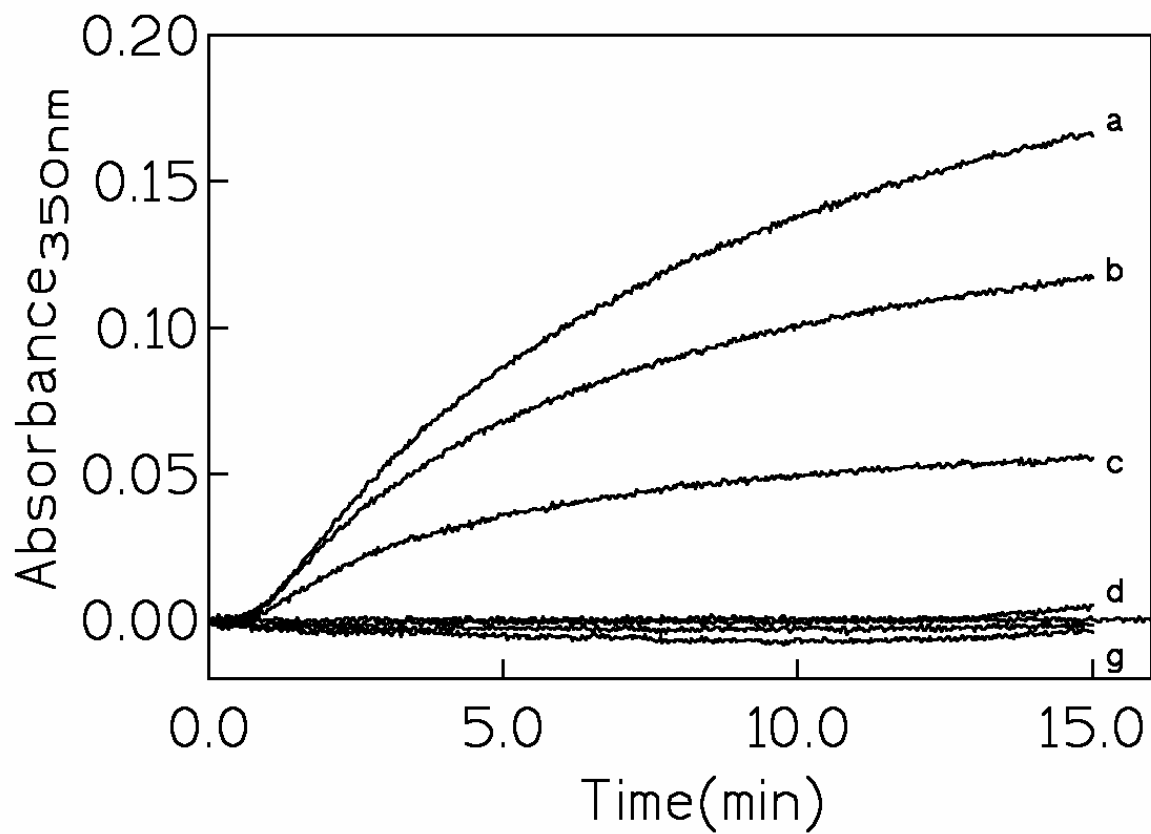


Figure 1B

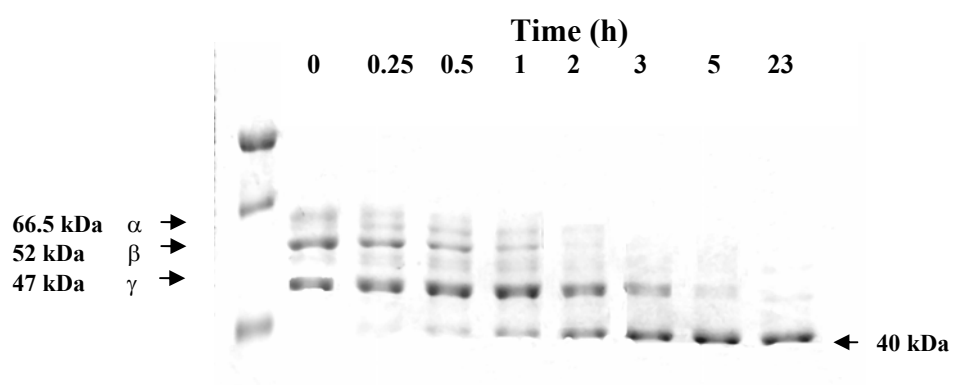


Figure 1C

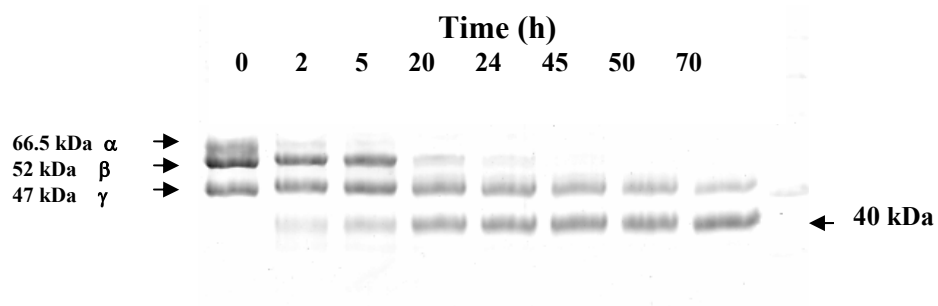
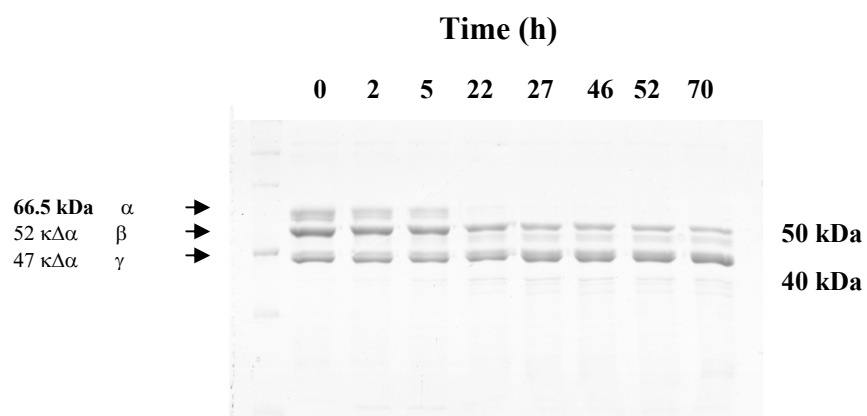


Figure 1D



**Figure 1E**

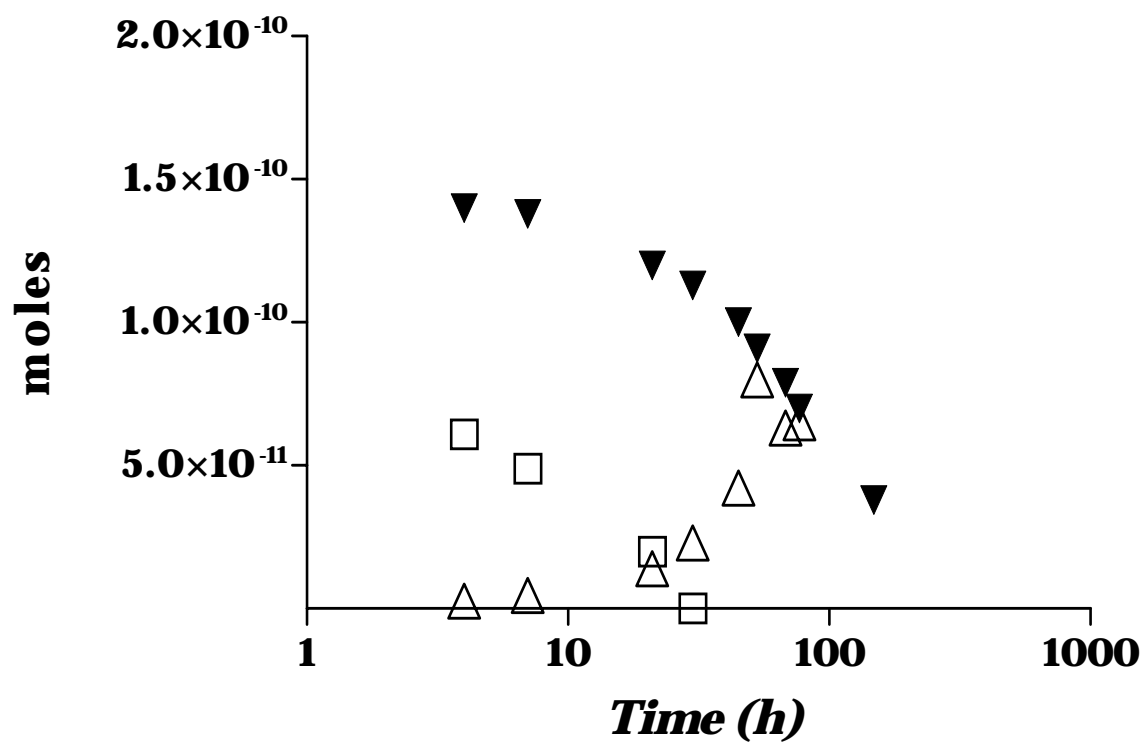


Figure 2A



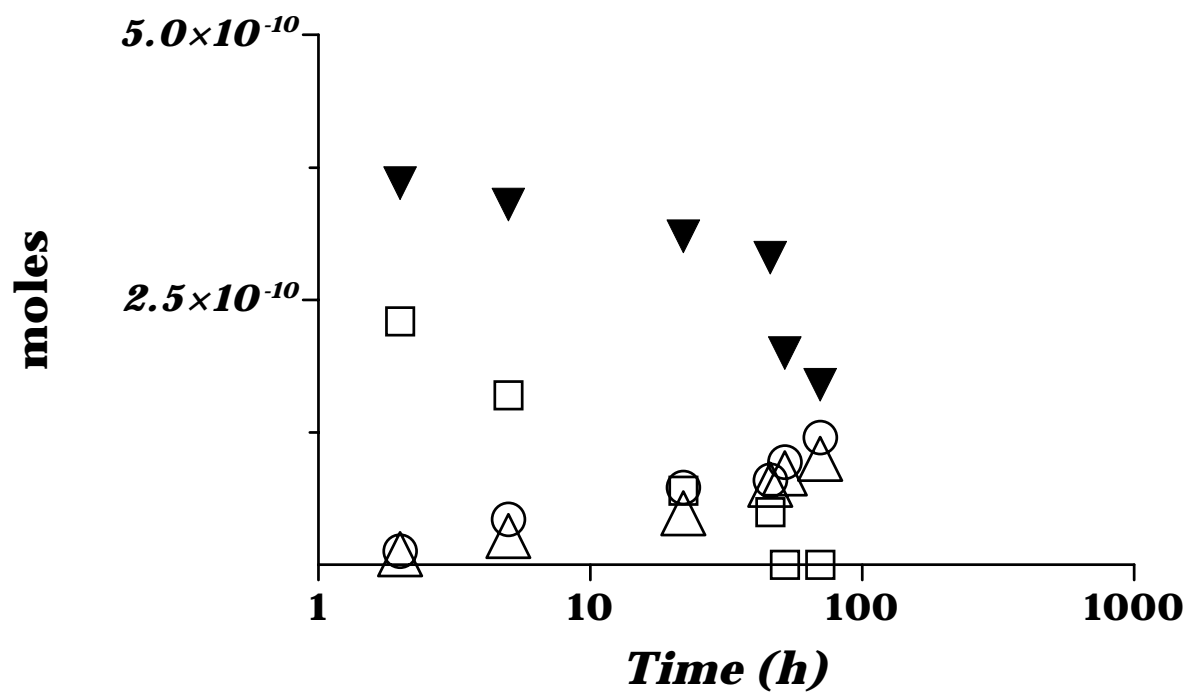


Figure 2B

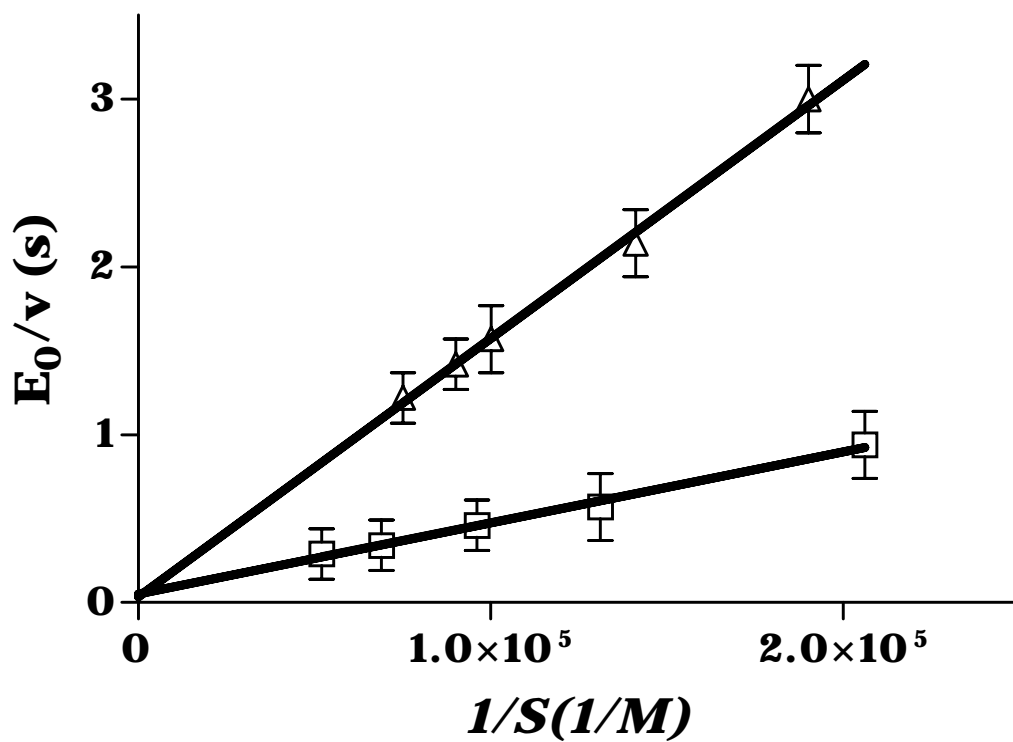


Figure 3A

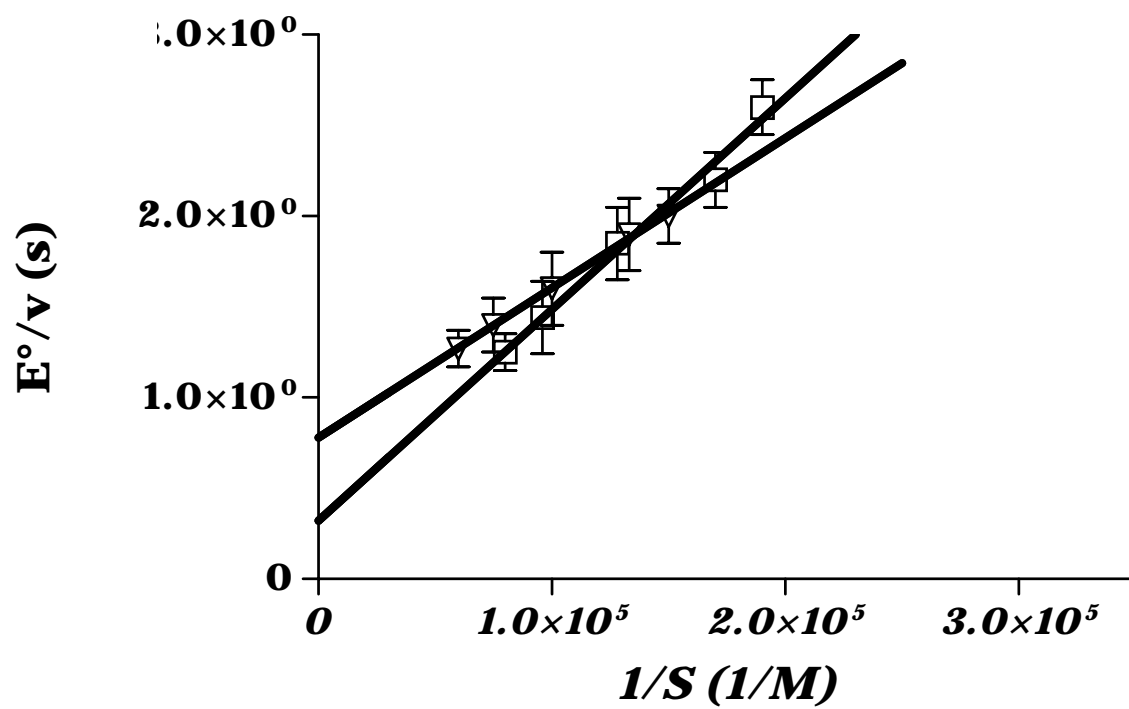


Figure 3B

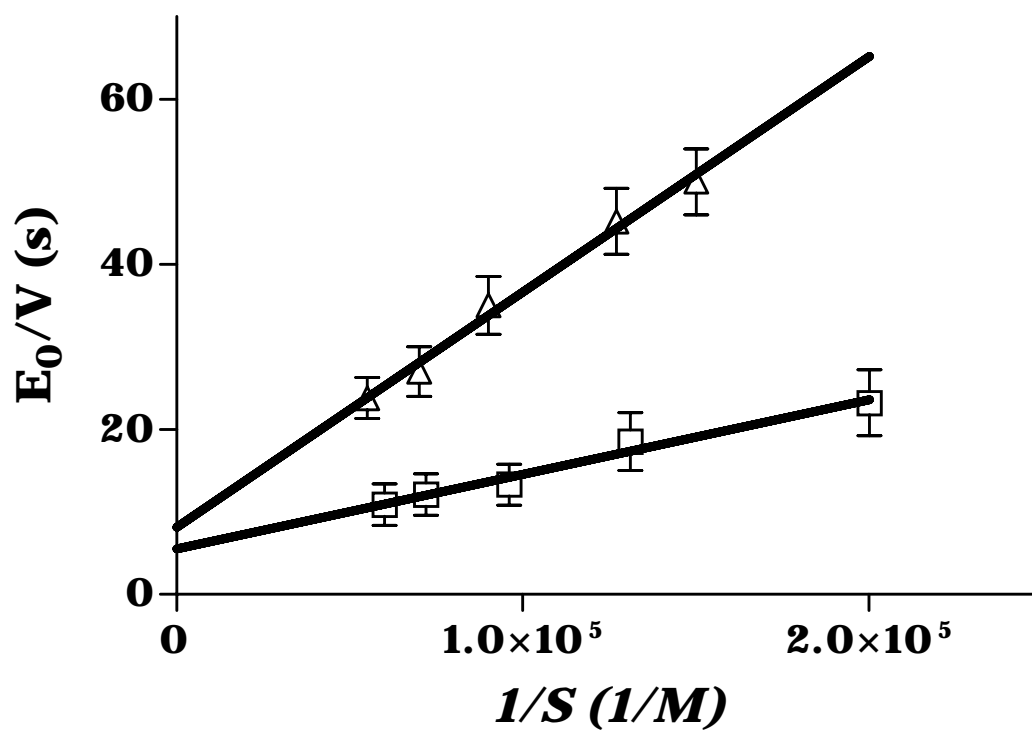


Figure 3C

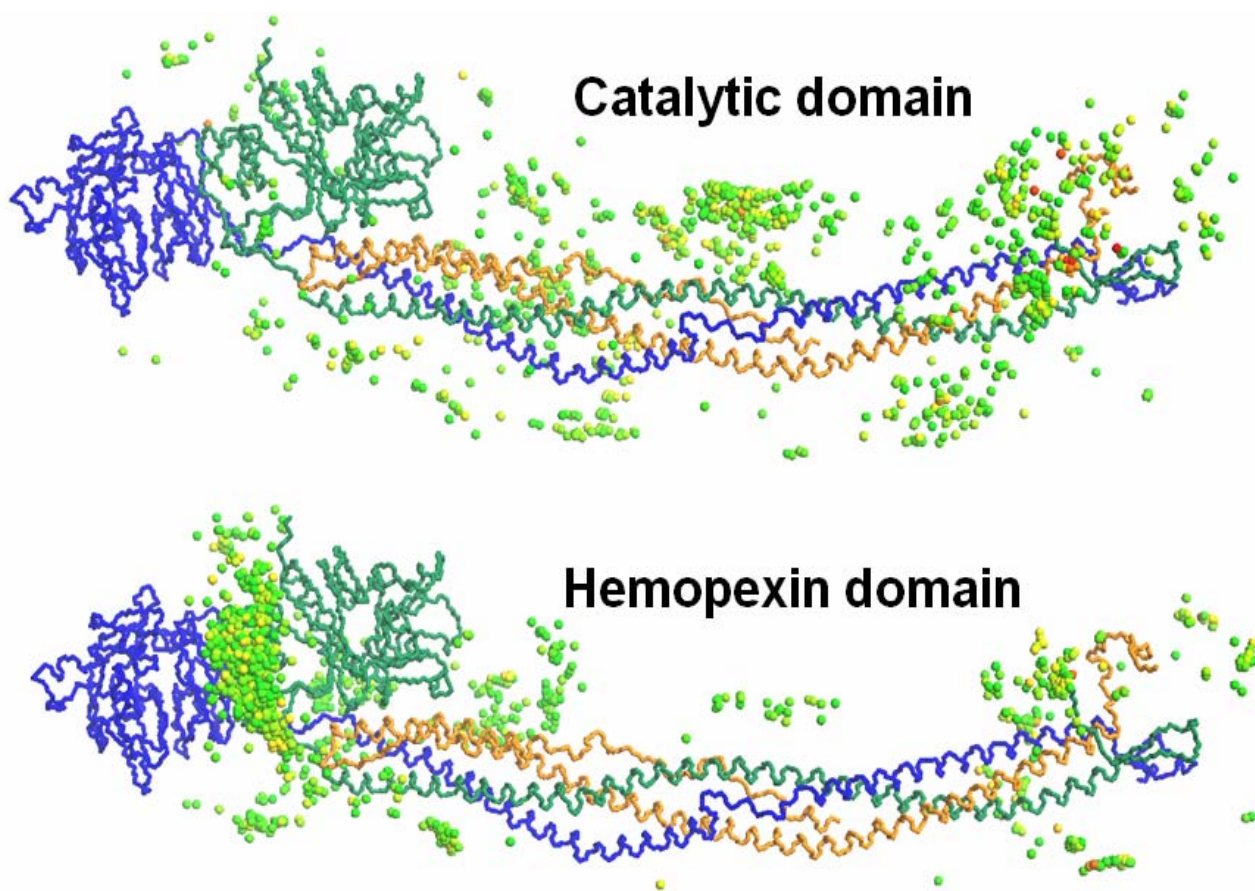


Figure 4A

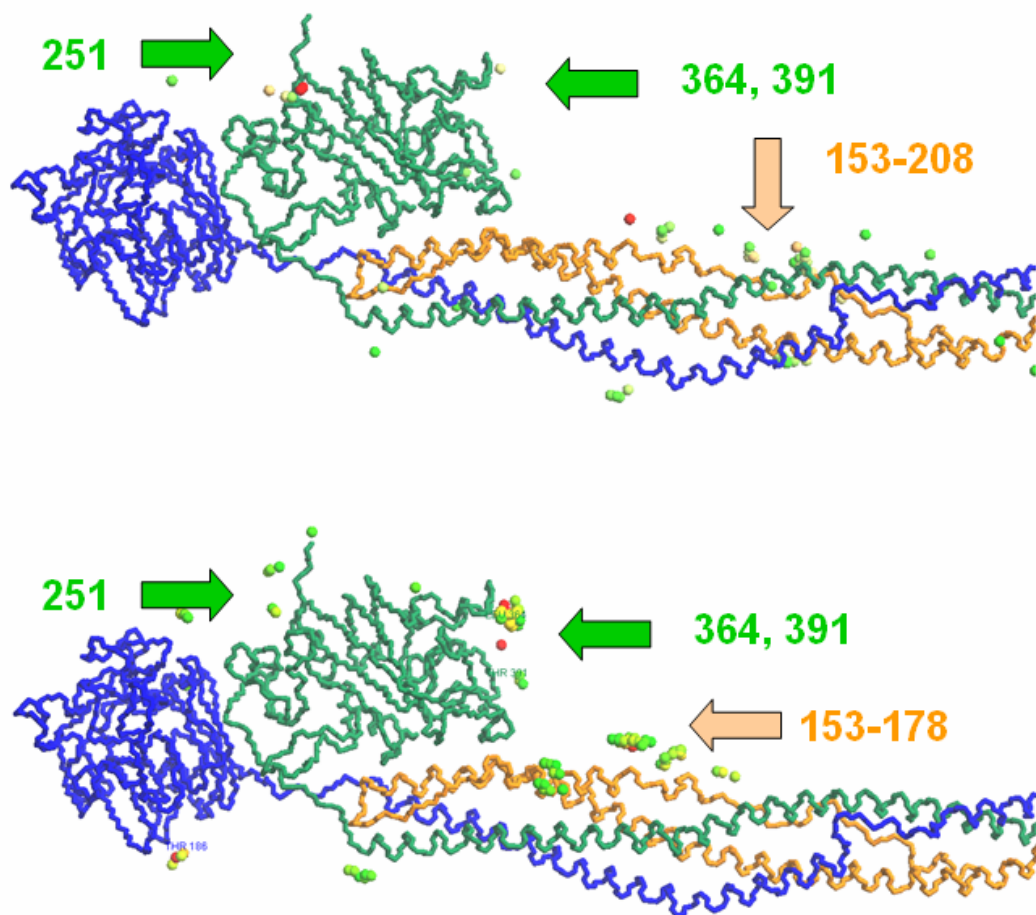


Figure 4B

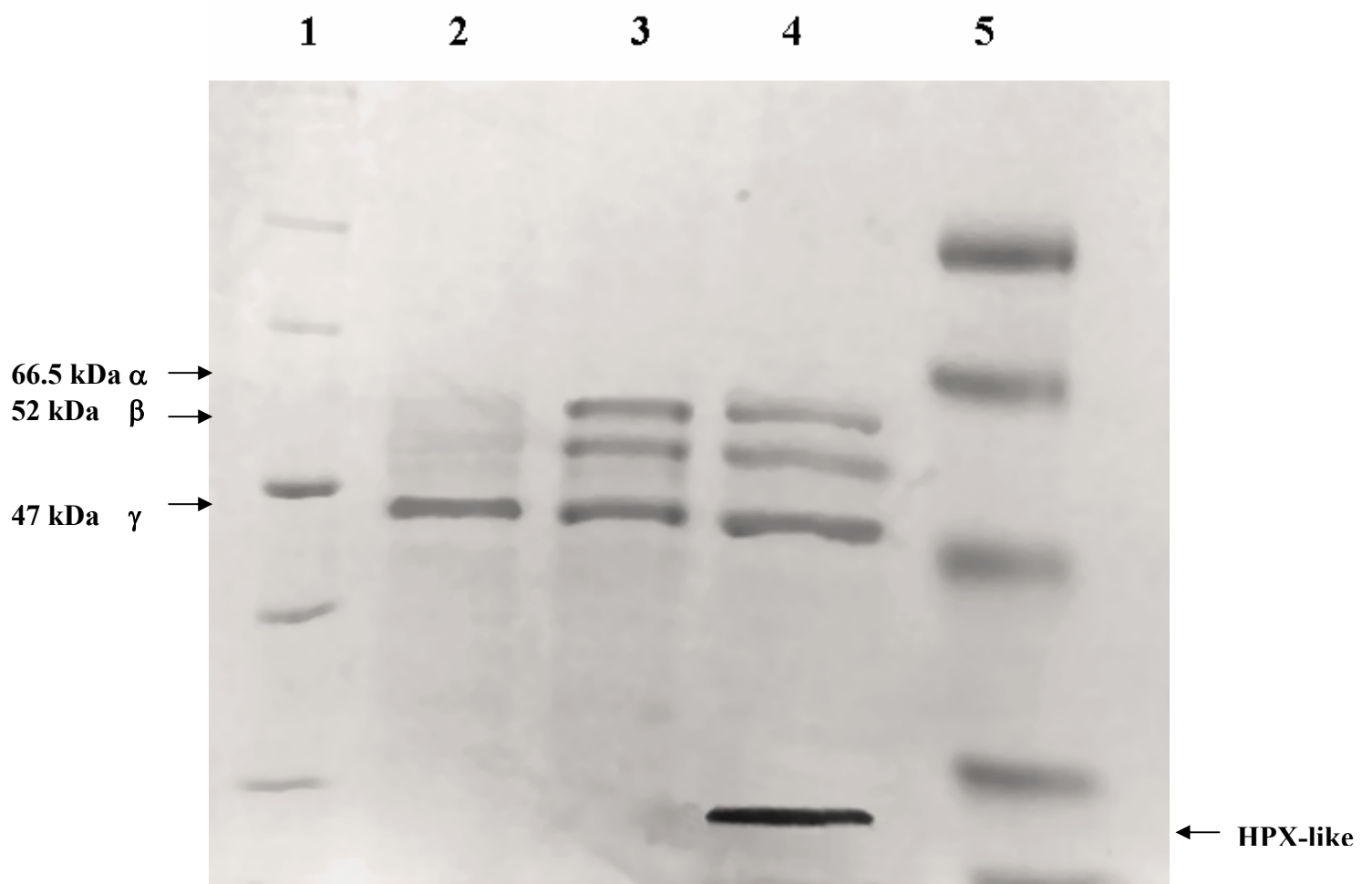


Figure 5

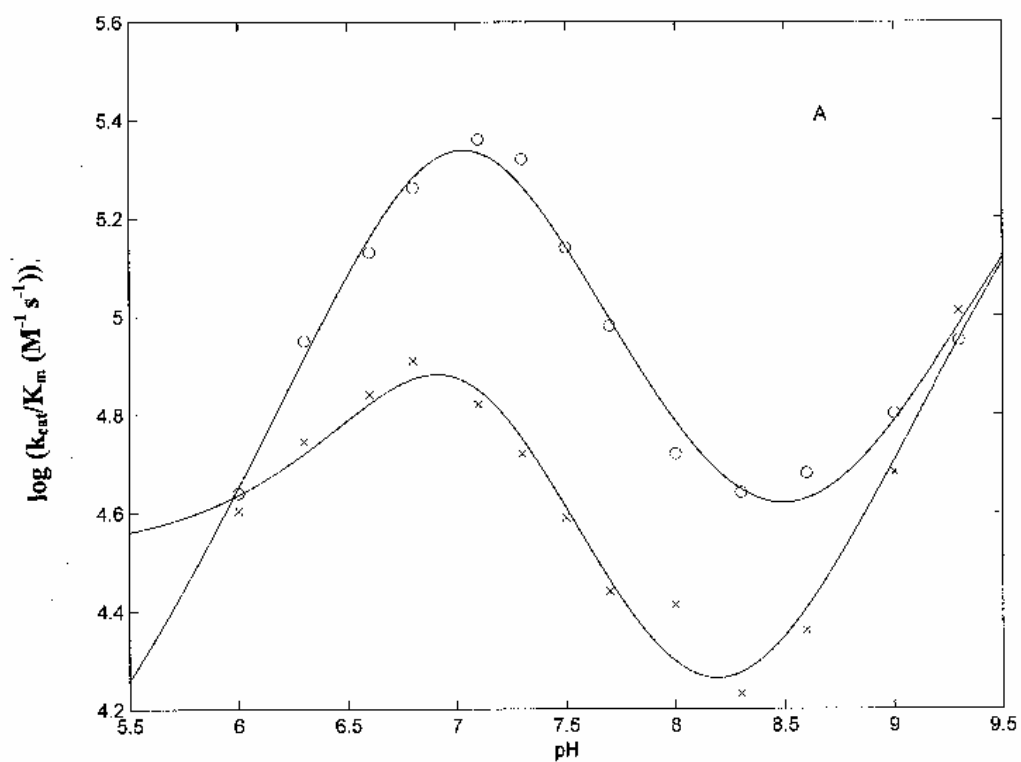


Figure 6A



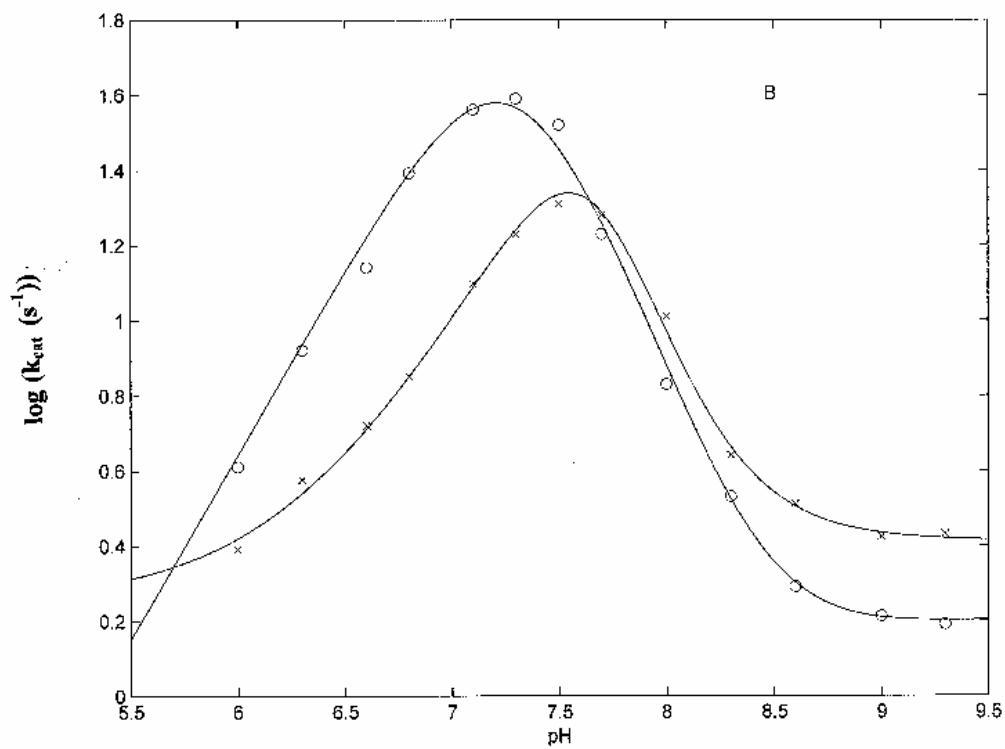


Figure 6B

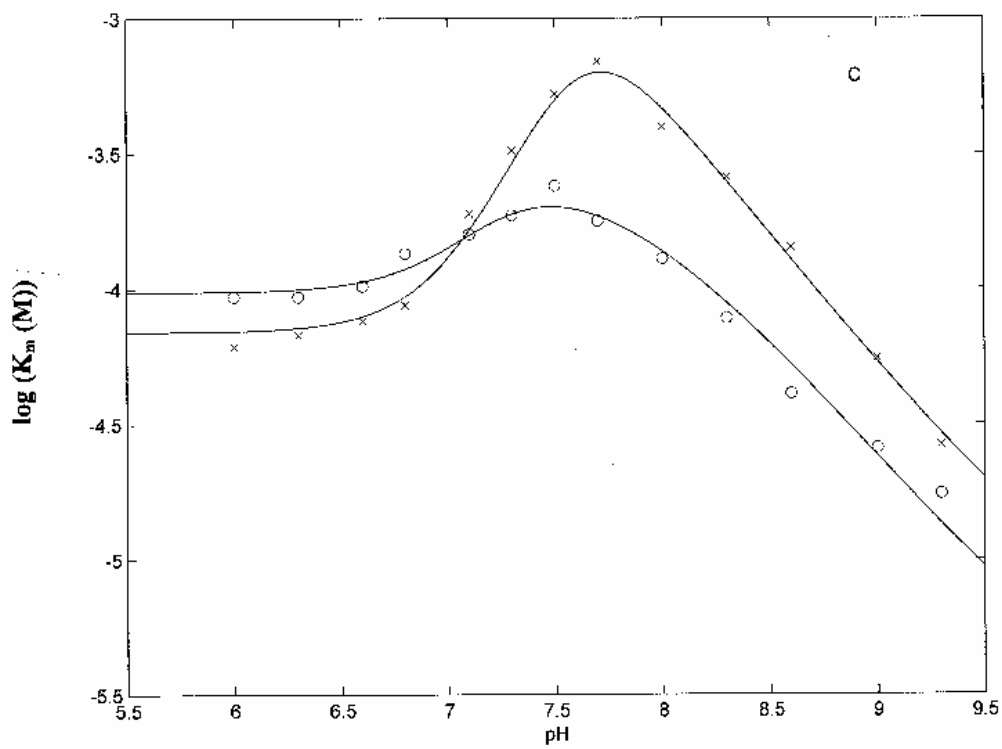


Figure 6C