Binding of Recombinant PrP^c to Human Plasminogen: Kinetic and Thermodynamic Study Using a Resonant Mirror Biosensor

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ABSTRACT **Transmissible spongiform encephalopathies are a class of sporadic, genetic and transmissible neurodegenerative diseases that affect both humans and animals. Propagation of these diseases is thought to be due to the misfolding of a neuronal glyco-protein, PrP^c, into a pathological insoluble conformer, PrP^{Sc}. In earlier works, some serum components were identified as exclusive PrP^{Sc}-interacting proteins (Fisher et al., Nature 2000;408:479), and thus those macromolecules were thought to represent a potential diagnostic endogenous factor discriminating between normal and pathological prion proteins. In contrast, in agreement with a recent work (Kornblatt et al., Biochem Biophys Res Commun 2003;305:518), in this paper we present a detailed thermodynamic and kinetic characterization of the interaction between recombinant bovine PrP^{c 25–242} and the human serum component plasminogen, measured using a resonant mirror technique: our results reveal a high-affinity interaction between the two binding partners. For comparison, the complex obtained from the purified full-length PrP^c and human plasminogen was also studied: both prion proteins (the recombinant bovine PrP^{c 25–242} and the purified full-length PrP^c) are able to bind human plasminogen. Both kinetic and thermodynamic parameters are affected by the modulation exerted by the H⁺ ions in solution. Moreover, the analysis of binding, according to canonical linkage relationships, suggests the involvement of a His residue, consistent with the interaction between other serine (pro)enzymes and their ligands. Proteins 2005;58:728–734.**
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Key words: prion protein; human plasminogen; biosensor; kinetics; thermodynamics; pH effect

INTRODUCTION

Transmissible spongiform encephalopathies (TSE), or prion diseases, are a class of fatal neurodegenerative disorders affecting both humans and animals. Most of them arise sporadically, some are genetically induced, and a minor ratio can be transmitted between mammals by dietary exposure to, or inoculation with infected tissues.[1]

Propagations of the disease are likely to rely only on a structural change in a neuronal glyco-protein,[2,3] PrP^c which contains two glycosylation sequons in its primary structure. In the disease, the native cellular form, PrP^c, is converted into its insoluble conformer, PrP^{Sc}, containing an increased proportion of β-sheet,[4] and a proteinase K-resistant core. PrP^{Sc} is thought to replicate by imparting its conformation onto PrP^c.[2] Prion diseases include bovine spongiform encephalopathies (BSE)[5] in cattle and scrapie in sheep.[6] In human prion diseases, such as the Gerstmann-Straussler-Scheinker (GSS) disease,[7] the kuru,[8] the fatal familial insomnia,[9] the sporadic and variant form of Creutzfeld-Jakob disease,[10,11] while the conversion of PrP^c to PrP^{Sc} and its accumulation seem to be fundamental events in disease propagation, other aspects of PrP expression, topology, folding and trafficking may be important in the pathological processes which result in disease. The functions of the prion protein are not yet clear, although possible roles in copper transport, synaptic function, circadian rhythm, signal transduction and promotion of genetic diversity have been proposed.[12–16] TSEs are characterized by a long incubation period, behavioral disturbances, and ataxia, and histologically by a vacuolation of neurons and the neuropil, reactive gliosis, and neuronal loss. In BSE, hydrated autoclaving pretreatment of brain sections before PrP immunolabeling has revealed widespread deposition of abnormal PrP in that organ.[17] Experimental evidence indicates that the aetiology and pathogenesis of TSEs are related at least in part to abnormalities in processing of PrP, constitutively expressed in most tissues but at particularly high levels in the central nervous system.[18] Because deposition of PrP^{Sc} alone is thought not to be a sufficient cause of neuropathology,[19] PrP^{Sc} probably damages the brain by interacting

with other cellular constituents. In published works, some serum components, expecially plasminogen, a serum pro-protease implicated in neuronal excitotoxicity,[20–22] were identified as a PrP^{Sc}-interacting protein and a PrP^c-non-interacting protein. For this reason plasminogen was thought to represent an endogenous factor discriminating between normal and pathological prion proteins. This property might be exploited for diagnostic purpose.[23] In order to better understand the nature of the interaction, in this work we report a detailed study performed with the IAsys resonant mirror biosensor, on the kinetic and ther-modynamic parameters characterizing the interaction be-tween human plasminogen and nonglycosylated recombi-nant PrP^c 25–242, over a wide range of pH.

In agreement with a recent work,[24] we show here that in these conditions rec PrP^c 25–242 interacts with immobilized plasminogen.

Moreover, as a validation, purified native full-length PrP^c was tested: the interaction analysis of both prion proteins with human plasminogen resulted in comparable equilibrium dissociation constants.

MATERIALS AND METHODS

Chromatographic Mono-Q (HR 5/5) column and HiTrap chelating column were obtained from Pharmacia-Amer-sham (Sweden). The CMD cuvette and the NHS immobili-zation kit, containing NHS, EDC and 1M ethanolamine (pH 8.5) were obtained from Affinity Sensors. Urea, NaOH, NaCl, Tris, KCl, CaCl$_2$, NaH$_2$PO$_4$, obtained from J.T. Baker, Tween-20, MES, CuSO$_4$, ZW 3-12, sucrose, human plasminogen, PNGase F, PMSF and TPCK obtained from Sigma-Aldrich were of analytical grade. Recombinant PrP^c bovine (25–242) and mouse monoclonal anti-PrP Antibody (mAB) 6H4 were kindly provided by Prionics. FPLC experi-ments were performed on an AKTA Chromatographic system obtained from Pharmacia-Amersham. Kinetic and thermodynamic studies were performed on an IAsys plus device, Affinity Sensors, obtained from ThermoFinnigan.

Purification of Plasminogen

Human plasminogen was irreversibly inactivated by adding PMSF using a standard procedure.[25] The resulting solution was injected on a Mono-Q column and eluted at 0.5 ml/min flow rate with the following segmented gradi-ent of A (Tris-HCl 50 mM, pH 9) and B (Tris-HCl 50 mM, NaCl 1 M, pH 9) buffers: 0–25% B in 8 min, 10 min at 25% B, 25-100% B in 20 min, 8 min at 100% B and finally 100–0% B in 4 min. The plasminogen elution profile shows a sharp single peak: the peak at 17 min, detectable both at 254 and 280 nm, corresponds to plasminogen (Fig. 1).

Purification of PrP^c

PrP^c was purified from bovine brain according to a previously published study.[26] Purity of the obtained pro-tein was assessed by standard Western Blot analysis performed with anti-PrP mAB 6H4. Final protein concen-tration was measured by the optical density at 280 nm using as extinction coefficient a value of 58718.0 M^{-1}cm^{-1}.[27]
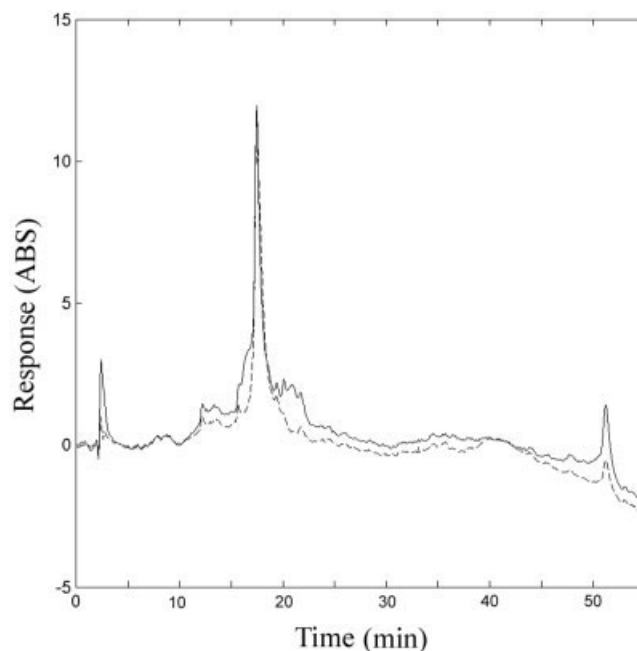

Fig. 1.   Elution chromatogram of plasminogen at λ = 254 nm (dashed line) and λ = 280 nm (continual line) (see Materials and Methods).

Deglycosylation of PrP^c

Native PrP deglycosylation was performed by overnight incubation with PNGase F at 37°C according to a standard procedure.[28]

Biosensor Studies

The CMD cuvette was rinsed with PBS-T (NaH$_2$PO$_4$ 10 mM, KCl 2.7 mM, NaCl 138 mM, Tween-20 0.05%$_{(v/v)}$, pH 7.4), and equilibrated with PBS (NaH$_2$PO$_4$ 10 mM, KCl 2.7 mM, NaCl 138 mM, pH 7.4) for approximately 10 min to ensure the establishment of a base line for the sensor trace at the set temperature (25°C). Plasmin-free plasminogen was covalently blocked onto the CMD matrix of an IAsys biosensor cuvette by the standard EDC/NHS coupling procedure.[29] The noncoupled ligand was removed by wash-ing with PBS buffer for 2 min. Remaining reactive groups were deactivated by a treatment with ethanolamine 1 M, which also assures the removal of any electrostatically bound material. This step was followed by a wash with PBS, a "pulse" wash with HCl 10 mM (regeneration buffer) and one last wash with PBS (Fig. 2).

The amount of immobilized ligand was calculated. A readout of about 500 arcsec was obtained. These conditions resulted in the coupling of a "Langmuir" partial monolayer of a protein of 80 kDa (1 ng/mm^2 $\approx$ 4 mg/ml). Then, the reaction environment was changed to the desired pH value, and rec PrP^c 25–242 was added at increasing concen-trations. These experiments were repeated at different pH conditions. Because repeated regenerations with HCl 10 mM were likely to affect the stability of immobilized plasminogen and its functional ability to interact with soluble rec PrP^c 25–242, regeneration steps were performed with PBS-T instead of HCl 10 mM: in fact, the presence of
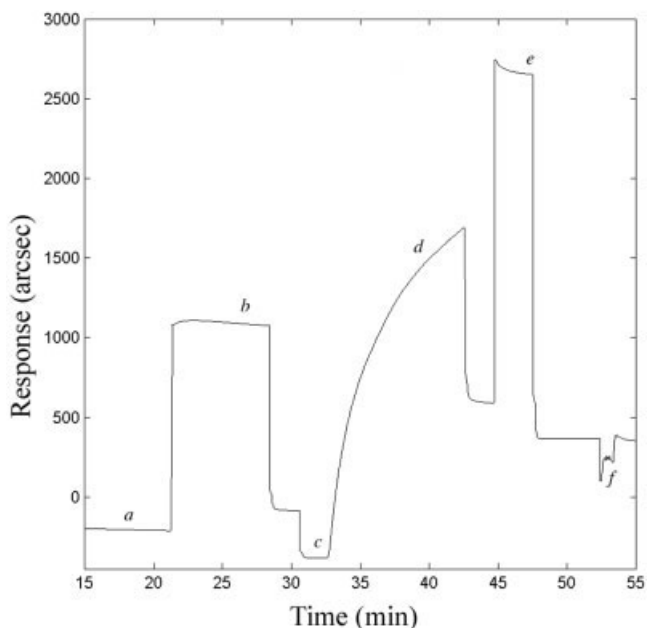
Fig. 2. Immobilization of plasminogen to CMD surface. PBS wash and baseline (**a**), activation with EDC/NHS (**b**), change to 10 mM acetate buffer (pH = 6.0) (**c**), addition of plasminogen (**d**), blocking with ethanolamine 1M (pH = 8.5) (**e**), wash with 10 mM HCl (**f**).
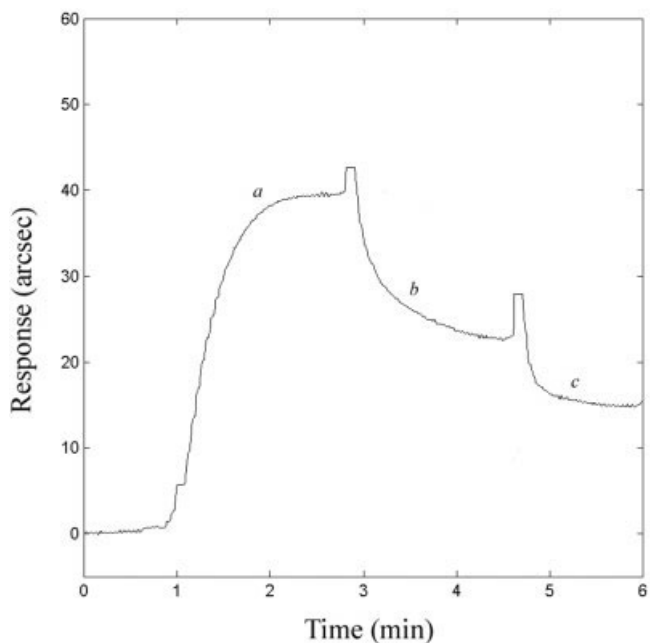

Fig. 3. Rec PrP$^{c\ 25-242}$ binding to immobilized plasminogen: monophasic association (**a**) and dissociation (**b**) curve, and regeneration (**c**).

a detergent is known to affect the binding properties of PrP^c [30] (Fig. 3).

Binding data were analysed using the "Fast Fit software" (Fison Applied Sensor Technology), supplied with the instrument; this program uses an iterative curve-fitting to derive the observed rate constant and the maximum response at equilibrium due to ligand binding at a certain ligand concentration.

RESULTS

Thermodynamics

The pH greatly affects the kinetic association and, less significantly, dissociation properties of the rec PrP$^{c\ 25-242}$-plasminogen recognition process. In fact, the association and dissociation kinetics measured in the range between pH 6.0–8.0 showed different time-courses.

The binding surface containing immobilized plasminogen was obtained as described in Experimental Procedures. The immobilization procedure was optimized after several experiments based upon the variation of plasminogen concentration and immobilization pH value. A plasminogen concentration value of 150 μg/ml and a value of 5.2 for the immobilization pH (chosen on the basis of the plasminogen isoelectric point) were found to best suit a good immobilization of plasminogen while maintaining its functional properties. Under these conditions a final read-out of about 500 arcsec, upon plasminogen immobilization, was obtained. These data were adequate, since immobilization levels should be fairly low for the particular binding studies analyses, in order to minimize steric hindrance and other effects complicating the kinetic analysis.

The binding experiment was repeated at increasing concentrations of rec PrP$^{c\ 25-242}$. At any titration step, baseline achievement was assessed before adding rec PrP$^{c\ 25-242}$, then the regeneration steps were achieved as described in Experimental Procedures. Each binding reaction was followed up to steady state (data not shown). In Figure 4, monophasic association and dissociation time-courses of rec PrP^c binding to immobilized plasminogen are reported.

Equilibrium dissociation constants were calculated for pH values between 6.0 and 8.0. Complex stability shows a bell-shaped curve between pH 6.2 and 7.8: pH dependence of the pK$_D$ shows a maximum at pH 7.2, values ten-fold greater than those measured at pH 6.2 and 7.8, where the curve shows two minima, while at more acidic (or more alkaline) pH values, the pH dependence of the dissociation equilibrium constant increases [Fig. 5(A)]. In addition, binding experiments between native PrP^c purified from bovine brain and plasminogen were performed at pH 7.4 [see Fig. 4(C, D)]. The measured thermodynamic equilibrium constant for the native PrP^c-plasminogen interaction was comparable ($K_{D,recPrPc} = [3.39 \pm 0.02]*10^{-8}$ M, $K_{D,nPrPc} = [1.49 \pm 0.02]*10^{-8}$ M) to that obtained for the rec PrP^c.

Kinetics

Calculation of the association (k_{ass}) and dissociation (k_{diss}) rate constants for rec PrP$^{c\ 25-242}$ binding to plasminogen further defines the mechanistic properties of the macromolecular recognition process. The association phase allowed the measurement of the kinetic association constant, while the fast dissociation phase contributed to the high standard deviation associated to the dissociation constant [Fig. 5(D)].

The standard deviations related to each kinetic association constant value k_{ass} (each value was calculated from at least five association/dissociation kinetic experiments) are
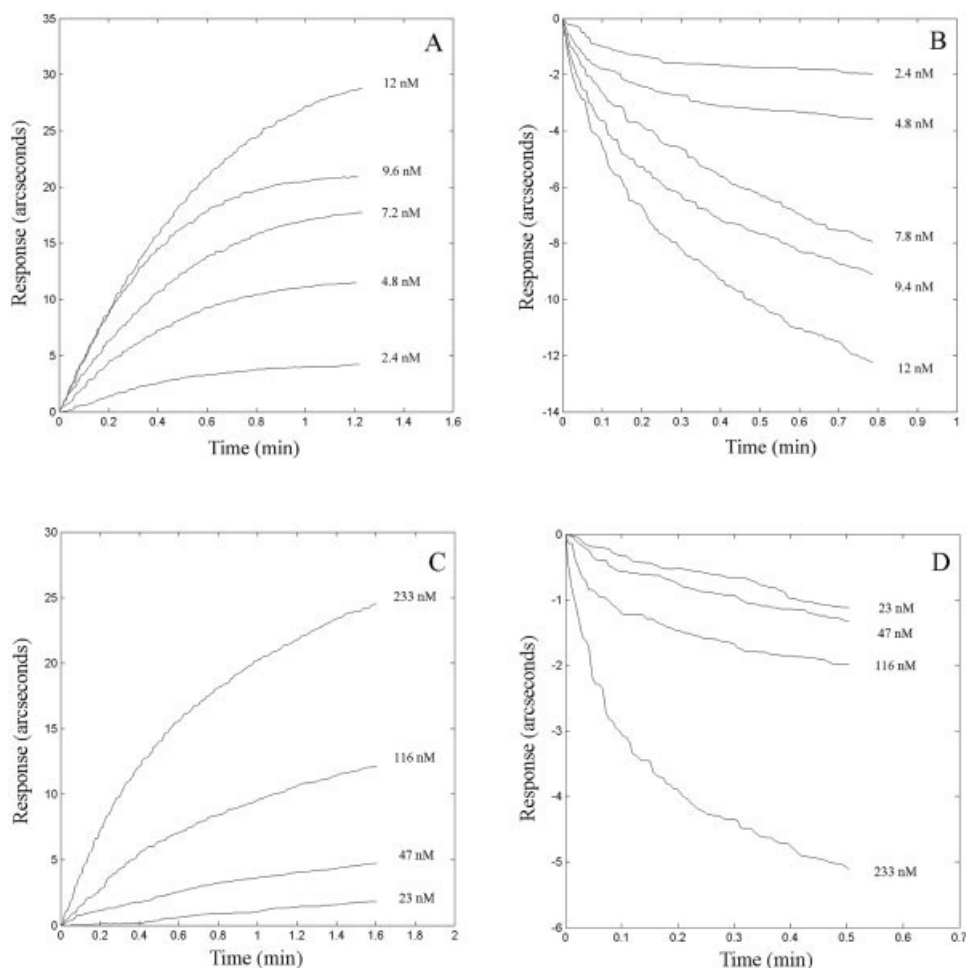
Fig. 4. Rec PrP^{c} $^{25-242}$ and native PrP binding to immobilized plasminogen: overlay of association and dissociation phases measured at increasing concentrations of rec PrP^{c} $^{25-242}$ (**A**, **B**) and native PrP^{c} (**C**, **D**), pH = 7.4.

negligible because of the high precision experimental raw data (the instrument short-term noise is less than 1 arcsecond).

Kinetic analysis of the binding of native PrP^{c} to plasminogen showed a different recognition behavior. These analyses revealed kinetic constant values lower than nonglycosylated rec PrP ($k_{ass,recPrPc}$ = [779275.0 ± 11302.9] M^{-1}s^{-1}, $k_{ass,nPrPc}$ = [98916.1 ± 134.0] M^{-1}s^{-1} and $k_{diss,recPrPc}$ = [2.64 ± 0.14]*10^{-2} s^{-1} , $k_{diss,nPrPc}$ = [1.48 ± 0.02]*10^{-3} s^{-1}). Such differences in terms of kinetic association rate constants could be mostly attributed to the large size of the N-linked oligosaccharides present in the native PrP^{c} surface (the probable glycosylation sites are the Asn 181 and Asn 197).[31]

The carbohydrate moieties in fact shield large exposed regions of the prion protein, covering orthogonal faces of the protein, sterically hindering intramolecular protein–protein interaction[1]: this fact could explain the lower association rate exerted by native PrP^{c} in comparison with rec PrP^{c} $^{25-242}$. On the other hand, the dynamic properties and the flexibility of the oligosaccharidic chains may provide a further anchoring site for the ligands and a route for the protein folding by allowing the protein to interact with other macromolecules, so that the decreased k_{diss} value could be in part explained. Finally, these combined

effects (negative) on the association and (positive) dissociation rates will cancel out and do not affect the thermodynamics of the complex.

The results of the analysis of the interaction between plasminogen and deglycosylated native PrP^{c} confirms the role of glycosylation on the kinetic and thermodynamic properties of the complex: in fact, such a complex was characterized by kinetic constant values comparable to those obtained for rec-PrP^{c} ($k_{ass, degly-nPrPc}$ = [613374.0 ± 14111.9] M^{-1}s^{-1}, $k_{diss, degly-nPrPc}$ = [6.97 ± 0.47]*10^{-3} s^{-1}), whereas thermodynamics are still not affected ($K_{D, degly-nPrPc}$ = [1.14 ± 0.08]*10^{-8} M).

The pH dependence of kinetic and thermodynamic constants of the complexes at constant ionic strength was analysed according to earlier studies.[32] The theoretical curves shown in Figure 5 were calculated with a nonlinear least-square curve fitting program, from Equations 1 and 2, based on canonical linkage relationships:[33–35]

$$\log k_{ass,obs} =$$

$$\log \frac{k_{ass,0} + k_{ass,1} (\lambda_1 + \lambda_3)[H^+] + k_{ass,2}\lambda_1\lambda_3[H^+]}{(1 + \lambda_1[H^+])(1 + \lambda_3[H^+])} \quad (1)$$

where the parameters $k_{ass,0}$, $k_{ass,1}$, $k_{ass,2}$ are respectively the rate constants for the unprotonated, single-protonated
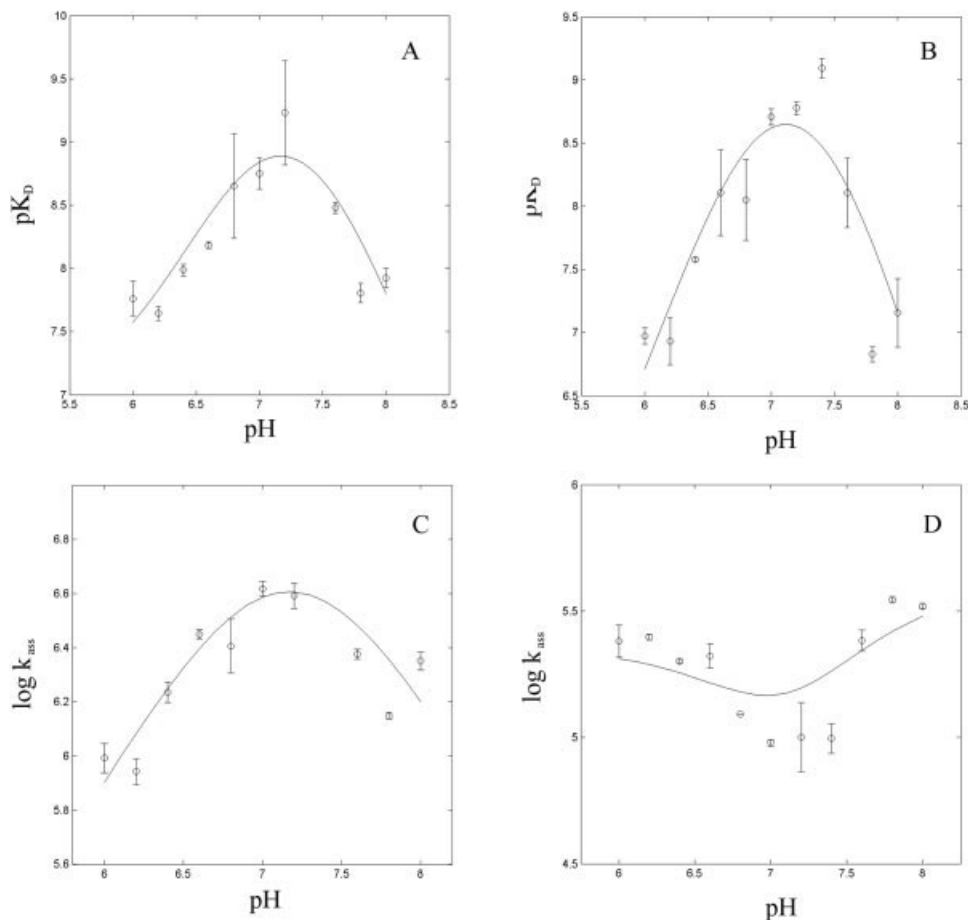
Fig. 5. pH dependence of equilibrium dissociation constants K_D and kinetic association rate constants for the recombinant (**A**, **C**) and native prion protein (**B**, **D**). The solid lines showed in panels A and B are drawn by using Equation 2, and the the solid line showed in C and D are drawn by using Equation 1; the parameters were obtained by nonlinear regression analysis of the experimental data.

and double-protonated species, and λ_1 and λ_3 are the first and the seconds proton binding constants for the free pro-enzyme.

$$\log K_{D,obs} = \log K_D - \log\left(\frac{(1 + \lambda_1 [H^+])(1 + \lambda_3 [H^+])}{(1 + \lambda_2 [H^+])(1 + \lambda_4 [H^+])}\right)^n$$

(2)

The bell-shaped pH-dependency of k_{ass} has a maximum around pH 7.0; it should be noticed that the two macromolecules associate less favorably at pH 6.2 and 7.8. The fast association rates over this pH range suggest that the plasminogen tethering onto the cuvette surface does not affect plasminogen binding to rec $PrP^{c\ 25-242}$.

A simple model for the interpretation of the pH dependence of the kinetic dissociation constants cannot be used; kinetic pH dissociation constant dependence is considerably low all over the pH range of interest and it shows its maximum in the pH range 6.0–6.6, [Fig. 5(C)]. In this pH range, k_{diss} resulted in a particularly high value: in these conditions the dissociation of the complex is kinetically promoted. This is the reason why, instead of a HCl 10-mM regeneration step (it could compromise the functionality or stability of the macromolecule blocked on the cuvette surface), we used a buffered PBS-T solution at pH 6.4:

Tween-20 increased the plasminogen surface regeneration steps.

The bell-shaped pH-dependency of K_D has a maximum around pH 7.4, whereas complex stabilty is considerably lower at pH 6.2 and 7.8.

DISCUSSION

The experimental results presented in this study show a high affinity interaction between soluble rec $PrP^{c\ 25-242}$ and immobilized plasminogen over a wide pH range, and thus are consistent with the work of Kornblatt et al.[24] The interaction is characterized by low equilibrium dissociation constants, mostly due to high association rate constants.

The pH-dependent change in molecular recognition both in k_{ass} and K_D values for rec $PrP^{c\ 25-242}$ binding to plasminogen is strictly reminiscent of that observed for the binding of macromolecular inhibitors (i.e., Kunitz- and Kazal-type inhibitors) to serine (pro)enzymes and thus may be described in similar terms.[36–42]

The reported data indicate that the different kinetic and thermodynamic parameters can vary substantially as a result of a contained structural perturbation occurring at the interacting surface, which alter the specific recognition contact between plasminogen and rec $PrP^{c\ 25-242}$.

The ionization phenomena that take place on the interface between the proenzyme and rec PrP^c $^{25-242}$ seem to be responsible for both the kinetic recognition process and complex stability. The pH dependency of the k_{ass} values for rec PrP^c $^{25-242}$-plasminogen complex formation may be attributed to the presence of protonable groups in the free pro-enzyme, characterized by a first proton binding constant $\lambda_1 = 7.24 \pm 0.22$ and a second proton binding constant $\lambda_3 = 7.20 \pm 0.22$. The calculated rate constants, $k_{ass,0} = 1221.01 \pm 56.07$ M^{-1}s^{-1}, $k_{ass,1} = 10964782 \pm 174583$ M^{-1}s^{-1} and $k_{ass,2} = 1321.03 \pm 109.76$ M^{-1}s^{-1}, revealed that the pro-enzyme single-protonated state is critical to association phase [Fig. 5(B)].

Moreover, the increase in the K_D values for rec PrP^c $^{25-242}$ binding to plasminogen on increasing the pH from 6.0 to 7.2, may be interpreted as reflecting the acidic pK-shift of a His residue from $\lambda_1 = 7.24 \pm 0.22$ in the free pro-enzyme, to $\lambda_2 = 6.16 \pm 0.20$ in the plasminogen-rec PrP^c $^{25-242}$ complex, most likely corresponding to the His-57 catalytic residue, as seen for other serine (pro)enzymes.[43–46] The measured acidic pK-shift of the His residue could reflect the burial of the proenzyme active site residue upon complex formation, with change in the local dielectric constant and hydrogen bonds formation.[33,38,47–49] The calculated λ_3 and λ_4 values ($\lambda_3 = 7.20 \pm 0.22$, $\lambda_4 = 8.65 \pm 0.16$) can be attributed to a terminal α-amino group.

In addition, the analysis of the pH-dependency of K_D for the native PrP^c binding to plasminogen revealed that the glycosylation does not affect considerably the stability of the complex all over the pH range of interest: in fact, the bell shaped pH-dependency of K_D, reminiscent of that observed for rec PrP^c $^{25-242}$ binding to plasminogen, may be interpreted in terms of similar pK-shifts from $\lambda_1 = 7.24 \pm 0.22$ in the free enzyme to $\lambda_2 = 6.16 \pm 0.20$ and from $\lambda_3 = 7.20 \pm 0.22$ to $\lambda_4 = 8.54 \pm 0.34$, with n = 4 [Fig. 5(B)].

On the other hand, glycosylation considerably affects the plasminogen-native PrP^c recognition process: in fact, the pro-enzyme single-protonated state seems not to favorably recognize native PrP^c ($k_{ass,1} < k_{ass,0}, k_{ass,2}$), with respect to rec PrP^c $^{25-242}$ [Fig. 5(D)]. In this work we focused on investigating the thermodynamic and kinetic properties of the interaction between serum component plasminogen and rec PrP^c $^{25-242}$ and to this end a resonant mirror biosensor technology has been used; to the best of our knowledge, this is the first time that a resonant mirror has been used to gain quantitative information on the macromolecular recognition process of bovine PrP^c to human plasminogen.

Moreover, in this study both non-glycosylated and glycosylated prion proteins have been used. The glycosyl moiety was thought to take part in the conformational stability of the PrP^{c50,51} and the interaction with plasminogen was proposed to be conformationally-dependent[23]: in this work we quantified the effect exerted by glycosylation on the kinetic properties of the interaction between PrP^c and human plasminogen.

Other systems showed a modulation on binding by glycosyl moiety, although there are no available data on the effects of glycosylation/deglycosylation on kinetic constants.[52–56]

Recombinant prion protein shows a functional behavior similar to deglycosylated native full-length prion protein, whereas protein glycosylated native full-length prion protein shows comparable thermodynamic properties, but as a result of the combined effect of decreased association and dissociation rate constants.

Furthermore, these results show the fine modulation of the rec PrP^c $^{25-242}$-plasminogen macromolecular recognition process exerted by H$^+$ ions. The acid–base equilibria involving amino acid residues at the interface between the pro-enzyme and rec PrP^c $^{25-242}$ affect the complex stability association constant in a canonical linkage fashion, while the dissociation constant pH-dependence reveals a singular behavior.

The results obtained could be used in the development of a new biosensor-based diagnostic test in Transmissible Spongiform Encephalopaties via human serum plasminogen as capturing ligand.

ACKNOWLEDGMENTS

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