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Interaction of ERp57 with calreticulin: Analysis of complex formation and effects of vancomycin

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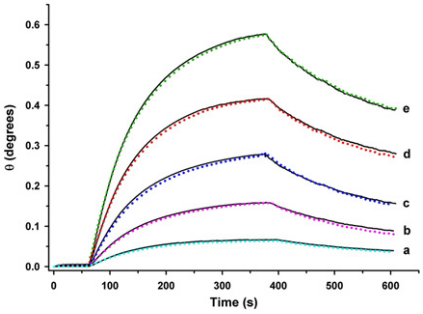
HIGHLIGHTS

- SPR data provide evidence for an ERp57 conformational change in the binding to CRT.
- Vancomycin could hinder the stabilizing conformational change in ERp57—CRT complex.
- Vancomycin decreases the amount of calreticulin on plasma membrane.

GRAPHICAL ABSTRACT

Sensorgrams for the affinity interaction of variable concentration of CRT to immobilized ERp57 in the presence of vancomycin. The concentrations of calreticulin (μM) were: 0.5 (a), 1.0 (b), 2.0 (c), 3.0 (d) and 4.0 (e).



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ABSTRACT

The protein ERp57 (also known as PDIA3) is a widely distributed protein, mainly localized in the endoplasmic reticulum, where it acts as disulfide isomerase, oxidoreductase and chaperone, in concert with the lectins calreticulin (CRT) and calnexin. The ERp57/CRT complex has been detected on the cell surface and previous studies have suggested its involvement in programmed cell death. Although the ERp57-CRT complex has been characterized, little is known about its role in different cellular compartments as well as inhibitors of this interaction.

We focused on the kinetic, extent and stability of the ERp57-CRT complex, using the surface plasmon resonance spectroscopy, investigating the possible role as inhibitor of the antibiotic vancomycin. Equilibrium thermodynamic data suggested that vancomycin may hinder the interaction between the two proteins and could interfere with the ERp57 conformational changes that stabilize the complex. Furthermore, by means of confocal microscopy, we evaluated the effect of the in vivo administration of vancomycin on the ERp57/CRT complex on the surface of HeLa cells.

The model presented here could be used for the search of other specific inhibitors/interactors of ERp57, which can be extremely helpful to understand the biological pathways where the protein is involved and to modulate its activity.

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1. Introduction

ERp57 (endoplasmic reticulum p57, EC 5.3.4.1, also known as PDIA3, ERp60 and GRP58) is a soluble protein, member of the disulfide isomerases family (PDIs), which is mainly located in the lumen of the endoplasmic reticulum (ER). Like the proteins belonging to the glucose-regulated proteins (GRP) family, ERp57 is overexpressed in response to glucose deprivation and to glycosylation blocking reagents [1], but it is also induced by many other cellular stress conditions, such as depletion of calcium stores from ER, presence of misfolded proteins, reductive stress, hypoxia, and low pH [2]. The location of ERp57 is not limited to the ER. In fact, it has been found in different subcellular compartments, including nucleus [3], cytoplasm [4] and cell surface membrane [5,6,7,8].

The role of ERp57 in the ER is relatively well understood. In this compartment, ERp57 acts as disulfide isomerase, oxidoreductase and chaperone, participating in the folding of newly synthesized glycoproteins, in concert with the lectins calreticulin (CRT) and calnexin [9].

In addition to the endoplasmic location, calreticulin has been localized in the cytoplasm [10], associated with the vitamin D receptor (VDR) together with other members of the steroid receptor protein superfamily [11] and involved in the nuclear export [12,13].

The function of the ERp57-CRT complex is well known in the ER, however the complex re-localization between different cellular compartments is still poorly understood. A recent research has revealed that calreticulin co-translocates to the cell surface in association with ERp57 [14]. It has been demonstrated that the exposure of calreticulin on the plasma membrane precedes anthracycline-induced apoptosis and that it is required for cell death to be perceived as immunogenic [15].

To date, a fully crystallographic structure of ERp57 is limited to a covalent complex with tapasin [16]. In the same report, the Authors suggested an in silico model for the luminal subcomplex of the peptide-loading complex, made up with tapasin, ERp57, MHC class I, and calreticulin. The ERp57-CRT complex has been characterized and it was demonstrated that the P domain of calreticulin binds the b and b′ domains of ERp57 [17, 18]. Moreover, Kimura et al. [19] have analyzed the ERp57-CRT interaction and demonstrated that ERp57 competitively forms complexes with PDI and CRT.

In this paper we focused the attention on the specific interaction between ERp57 and calreticulin. The kinetic and affinity of the binding of recombinant ERp57 to calreticulin has been investigated by surface plasmon resonance (SPR) spectroscopy. The SPR technique has recently demonstrated to be an interesting tool in biomolecular interactions, providing kinetic information along with affinity data which can be employed for thermodynamic studies [20, 21]. The extent of complex formation as a function of the temperature has been also evaluated, enabling the calculation of both equilibrium and transition-state thermodynamic parameters for this fundamental molecular recognition process. Considering that the physiological role and functions of ERp57 within the cell are not fully elucidated, the identification of specific inhibitors capable to perturb the ERp57 interactions, such as the ERp57-CRT complex formation, is highly desirable. It has been previously reported that ERp57 interacts with some antibiotics which interfere with its activity. In particular, vancomycin has been shown to bind reversibly to ERp57 and to inhibit the reductase and the DNA-binding activities of the protein [22]. In this context, the possible role of vancomycin as inhibitor of the ERp57-CRT interaction and its effect on the stability of the protein complex have been also investigated.

The experimental model presented here can be exploited for the detection of other specific inhibitors of ERp57, which could be used with greater effectiveness in vivo.

2. Materials and methods — experimental procedures

2.1. Materials

Human recombinant ERp57 was obtained as previously described [23]. Mouse monoclonal anti-ERp57 (sc 23886) and rabbit polyclonal anti-calreticulin antibodies were from Santa Cruz. Vancomycin and calreticulin were from Sigma.

2.2. SPR binding experiments

SPR experiments were performed with Eco Chemie Autolab SPR system (Ecochemie, The Netherlands). ERp57 protein was covalently coupled to a CMD20 SPR sensor chip (Xantec Bioanalytics GmbH, Munster, Germany) through its free amino groups using a mixture containing 0.5 mM ethyl(dimethylaminopropylcarbodiimide) (EDC) and 0.1 mM N-hydroxysuccinimide (NHS). To prepare a high-capacity surface, a 50 μl aliquot of ERp57 solution (0.1 mg/ml) was used.

Measurements of rate and extent of interaction between the immobilized protein and calreticulin over a concentration range were performed by monitoring the changes in the resonance angle shift at the SPR surface. Signals were collected at temperatures ranging from 10 to 35 °C and in the presence of 10 mM Tris–HCl, pH 8.0, as coupling buffer. The data were corrected for changes in the refractive index because of solvent mismatches, injection noise as well as non-specific peptide binding to the ligand-free surface.

For the study of interaction between ERp57 and vancomycin, the latter was covalently immobilized on a mixed self-assembled monolayer (SAM) on gold generated from the (2-(2-(2-(11-mercaptoundecyl-oxy)-ethoxy) ethoxy) ethyl alcohol, $(HS(CH_2)_{11}(OCH_2CH_2)_3OH)$, **1**, and the (2-(2-(2-(2-(2-(2-(2-(11-mercaptoundecyl-oxy)ethoxy)ethoxy)ethoxy)ethoxy) ethoxy) ethoxy) acetic acid, $(HS(CH_2)_{11}\text{-}(OCH_2CH_2)_6OCH_2CO_2H)$, **2** (Scheme S1, Supplementary data). A solution 1 mM of vancomycin was added on the modified surface, after pre-activation of the carboxylic groups with a mixture containing 0.5 mM EDC and 0.1 mM NHS in PBS for 30 min. The modified surface was incubated for 1 h with insulin at 4 °C. After being rinsed with deionized water, the substrates were blown dry under N_2 and used for the SPR measurements. For more details about the synthesis of compounds **1** and **2**, and surface modification see Supplementary data.

2.3. Cell culture

Human cervical adenocarcinoma (HeLa) cells were grown at 37 °C and 5% CO_2 in DMEM medium, supplemented with 10% (v/v) fetal bovine serum, 1% sodium pyruvate, 2 mM glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin.

2.4. Immunofluorescence analysis

HeLa cells were grown on glass coverslips and treated with 100 nM, 500 nM and 1 μM vancomycin for 10 min at 37 °C or left untreated as a control. Cells were washed with serum-free medium and PBS, then fixed in 4% paraformaldehyde for 20 min. In parallel, additional untreated control cells (C*) and 500 nM vancomycin treated cells (500*) were permeabilized with 0.1% Triton X-100 in PBS. Both non-permeabilized and permeabilized cells were then washed with 0.5% BSA in PBS, and incubated for 1 h in 1% BSA in PBS (PBS/BSA). To detect calreticulin, cells were incubated for 1 h with anti-calreticulin (rabbit polyclonal antibody) (1:30 dilution in PBS/BSA), washed in PBS/BSA, and incubated for 1 h with TRITC-conjugated goat-anti-rabbit-IgG (Jackson Immunoresearch) in the dark. To detect ERp57, cells were washed and stained with anti-ERp57 (mouse monoclonal antibody) (1:30 dilution) followed by washing and incubation for 1 h with FITC-conjugated anti-mouse-

IgG (Amersham) in the dark. Finally, coverslips were mounted in Vectashield (Vector Laboratories) and analyzed by a Leica DM-IRE2 confocal microscope. Images were recorded and processed by ImageJ 1.37v (LSC_lite Software). As negative controls, immunostaining was performed with no primary antibody incubation.

3. Results

3.1. Analysis of ERp57-CRT complex formation

ERp57 was covalently coupled to a carboxy-methylated dextran surface and tested for calreticulin binding. Data were collected in two different types of assay by SPR spectroscopy, thus allowing the detection of K_C and K_S affinity constants. K_C is the apparent binding constant for the interaction at the SPR sensor surface and was obtained by fitting on one-site binding isotherm the equilibrium SPR binding signals of solutions containing increasing concentration of calreticulin. K_S, which is defined as the thermodynamic binding constant for biomolecular binding in solution, was derived from a competition experiment by analyzing the SPR equilibrium signal with solutions containing a fixed amount of calreticulin and increasing concentration of ERp57.

The association and dissociation reaction between different concentration of free calreticulin and ERp57, immobilized on the surface, is reported in Fig. 1. The SPR signal upon binding as a function of time contains kinetic information, and this allows a full kinetic characterization of the binding. A comprehensive fitting analysis was performed with different kinetic models. The kinetic analysis of ERp57-CRT binding was not well fitted by a 1:1 Langmuir binding model even if we included a transport step for diffusion from the bulk to the sensor surface (Figure S1, Supplementary data), as indicated by the large value of the goodness of fit (Table S1, Supplementary data). As shown in Fig. 1A, a significantly improved fit was obtained using a two-step binding model described by the following equations [24,25]:

$$A_{bulk} \xrightarrow{k_t} A_{sensor} \qquad A_{sensor} + B \underset{k_{d1}}{\overset{k_{a1}}{\rightleftharpoons}} AB \qquad AB \underset{k_{d2}}{\overset{k_{a2}}{\rightleftharpoons}} (AB)^*.$$

The equilibrium constants of each step are $K_1 = k_{a1}/k_{d1}$ and $K_2 = k_{a2}/k_{d2}$ and the overall equilibrium binding constants are calculated as $K_A = K_1(1 + K_2)$ and $K_D = 1/K_A$. In this model, the analyte (A) binds to the ligand (B) to form an initial complex AB, which then undergoes a conformational change to form a more stable complex (AB)*. The kinetic information contained in the SPR curves was analyzed according to the above defined binding model using the program CLAMP [26].

This two-step model better describes the biphasic nature of the binding curve of calreticulin to ERp57, where an initial rapid increase of the signal is followed by a slower increase before reaching the equilibrium. In this model, binding becomes tighter after the conformational change. Changing the injection time revealed that the dissociation rate was progressively decreased after longer contact time, indicating that the stability of the initial ERp57-CRT complex increases over time (Figure S2, Supplementary data). This finding supports the conformational change model.

The binding responses of calreticulin, in the concentration range 0.5–4.0 μM, to the immobilized ERp57 are shown in Fig. 1B. The concentration of free calreticulin was corrected for small depletion of ligand due to binding. The SPR signal increased with the concentration of calreticulin during the association phase and then the signal decreased during the dissociation phase. The kinetic information contained in the SPR curves were analyzed according to the two-step binding model, with an estimated K_C value of 2.2 ± 0.5 μM.

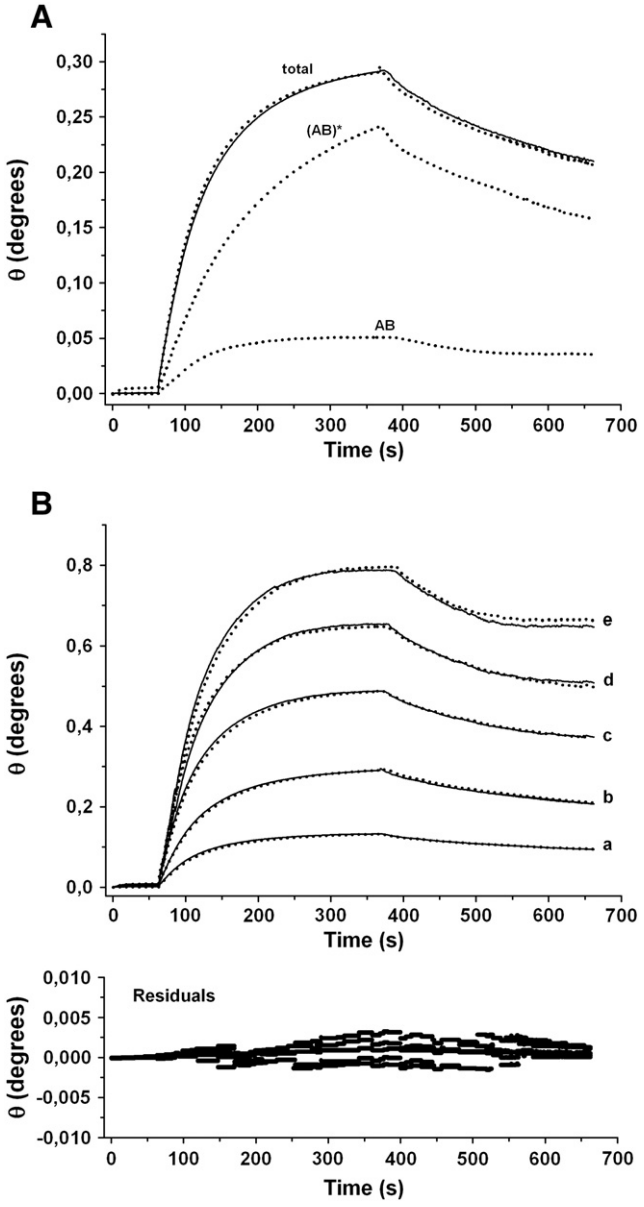

Fig. 1. (A) Sensorgrams of the binding of 1 μM CRT to ERp57 (immobilized onto the sensor surface) at 25 °C in 10 mM Tris–HCl buffer, pH 8.0. Experimental data (solid line) were fitted with the two-step binding model (dotted lines). Simulated curves displaying the initial binding AB and subsequent binding or conformational change (AB)* are the additive component from the fitted total curve. (B) Sensorgrams and residuals from the fit using a two-step binding model for the affinity interaction of variable concentration of CRT to immobilized ERp57 at 25 °C in 10 mM Tris–HCl buffer, pH 8.0. The concentrations of calreticulin were (μM): 0.5 (a), 1.0 (b), 2.0 (c), 3.0 (d) and 4.0 (e). The dotted curves are the fits to the experimental data according to the two-step binding model.

As reported in Fig. 2, the presence of free ERp57 in solution reduces the concentration of free calreticulin (competitive binding), resulting in a decrease of the equilibrium SPR signal. The competitive curve was fitted as described previously [27], and a K_S value of 2.8 ± 0.3 μM was obtained.

While K_C might be influenced by artifacts related to the carboxymethylated dextran surface of the SPR sensor, K_S is a thermodynamic binding constant derived from the interaction in solution, which in principle is independent from matrix surface effects. Interestingly, the value of K_C and K_S are not significantly different, demonstrating that the affinity of calreticulin for ERp57 at the sensor surface is identical to that in solution and K_C is not influenced by the sensor environment. Nevertheless, it is precisely the value of the solution-surface

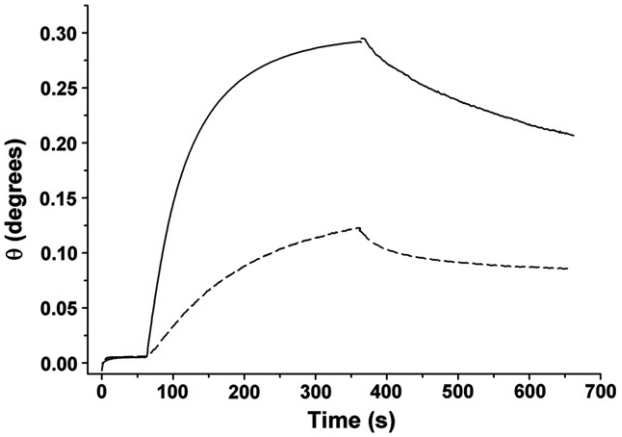

Fig. 2. Sensorgrams for the interaction between 1.0 μM of calreticulin and ERp57 (immobilized onto the sensor surface) in absence (solid line) and in the presence of 0.1 μM of ERp57 in solution (dashed line).

binding constant that is of interest in considering the in vivo environment for many receptor-ligand interactions. In order to bring the in vitro studies in cellular setting, we considered more suitable to use the value of K_C in the analysis of the ERp57-CRT interaction.

Table 1 summarizes the kinetic rate constants and the derived affinity constants for the interaction between calreticulin and ERp57 obtained using the two-state binding model. The K_D value reported in Table 1 is similar to those obtained by fitting the equilibrium SPR signal to a one-site binding isotherm (K_C) and the thermodynamic constant (K_S) derived from the biomolecular binding in solution, further validating the two-state binding model. Moreover, results on ERp57-CRT interaction provided values of affinity constants consistent with data already reported in the literature by NMR or ITC experiments [17].

A further aspect relates to the effect of different loading of ERp57, immobilized on the sensor surface, on the binding with CRT in solution. The standard deviation of the residuals of the global fitting analysis suggested that the ERp57-CRT complex formation is not affected by the amount of ERp57 immobilized on the surface (Figure S3).

In a control experiment, injection of CRT at different concentration over the CMD-modified surface (no ERp57 immobilized) resulted in a slight increase of the SPR signal with time (Figure S4). When the solution of CRT was replaced by buffer, a fast drop in the value of the Δθ back to its original value was observed, suggesting that there was no observable nonspecific binding to the CMD-modified surface.

Finally, the pH dependence of the binding response for ERp57-CRT interaction at 20 °C was analyzed and a maximum value was observed at pH 8.0 (Figure S2, Supplementary data).

3.2. Effect of vancomycin on ERp57-CRT complex formation

In order to investigate the influence of vancomycin on the binding parameters of ERp57-CRT interaction, we have conceived a SPR experiment monitoring the interaction between CRT and ERp57 (immobilized on the sensor surface) previously saturated with vancomycin. On such experiment is crucial to operate the protein injection (i.e. CRT) only after the ERp57 binding sites have been saturated with vancomycin. Furthermore, it is necessary to keep constant the equilibrium between ERp57 and the saturating vancomycin during the injection process, in order to assure that the SPR response is caused by the interactions with CRT. The experimental results and the best fitting curves are shown in Fig. 3 and the kinetic parameters summarized in Table 1.

3.3. Thermodynamic analysis of ERp57-CRT binding

The equilibrium constants at different temperature were analyzed using the integrated van't Hoff equation to obtain a plot of ln K_D versus 1/T (Figure S6, Supplementary data).

Non-linear regression yielded the following parameters for the ERp57-CRT complex at 25 °C: $\Delta H^o = (-7.4 \pm 0.6)$ kcal mol^{-1}, $T\Delta S^o = (-1.3 \pm 0.2)$ kcal mol^{-1} and $\Delta G^o = -6.1$ kcal mol^{-1}. The convexity of the curve indicates a negative heat capacity: $\Delta C_p = (-14 \pm 3)$ kcal mol^{-1} K^{-1}.

The change of free energy (ΔG^o), calculated from the affinity at each temperature, provides a measure of the stability of the complex between calreticulin and ERp57. The relative contributions of each step to the overall free energy of binding were estimated from the affinity constants for the two individual steps. The first binding step contributed most of the overall free energy of binding ($\Delta G^o{}_1 = -7.0$ kcal mol^{-1}; $\Delta G^o{}_2 = -0.5$ kcal mol^{-1}).

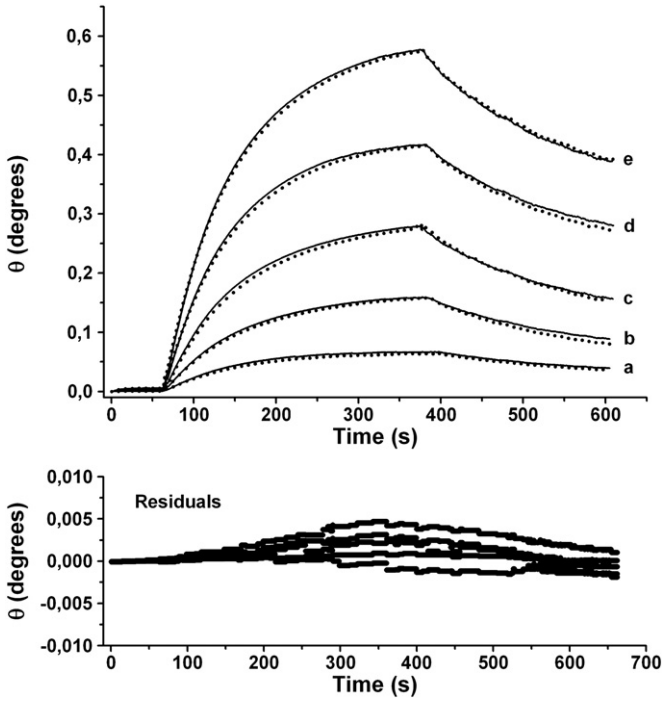

Fig. 3. Sensorgrams and residuals from the fit using a two-step binding model for the affinity interaction of variable concentration of CRT to immobilized ERp57 at 25 °C in 10 mM Tris–HCl buffer pH 8.0 in the presence of 45 μM vancomycin. The concentrations of calreticulin were (μM): 0.5 (a), 1.0 (b), 2.0 (c), 3.0 (d) and 4.0 (e). The dotted curves are the fits to the experimental data according to the two step binding model.

Table 1
Kinetic parameters for the binding interaction between calreticulin and ERp57 in absence and in presence of vancomycin.

	k_{a1} 10^4 M^{-1} s^{-1}	k_{d1} 10^{-2} s^{-1}	k_{a2} 10^{-2} s^{-1}	k_{d2} 10^{-3} s^{-1}	K_1 10^4 M^{-1}	K_2	K_D μM
ERp57-CRT	16 ± 0.4	12 ± 1	0.8 ± 0.2	4.2 ± 0.6	13	1.9	2.6
ERp57-CRT vancomycin	0.1 ± 0.3	0.4 ± 0.8	0.3 ± 0.2	3.2 ± 0.6	2.5	1.0	20

Table 2
Parameters for activation of the transition state of ERp57-CRT in the presence and absence vancomycin.

	$\Delta G_a^{\ddagger}$ (kcal mol^{-1})	$\Delta H_a^{\ddagger}$ (kcal mol^{-1})	$T\Delta S_a^{\ddagger}$ (kcal mol^{-1})	$\Delta G_d^{\ddagger}$ (kcal mol^{-1})	$\Delta H_d^{\ddagger}$ (kcal mol^{-1})	$T\Delta S_d^{\ddagger}$ (kcal mol^{-1})
ERp57-CRT	11.4	-4.6 ± 0.5	-16 ± 1	19	4.9 ± 0.4	-14 ± 1
ERp57-CRT vancomycin	17.3	-1.4 ± 0.5	-19 ± 1	23.1	6.1 ± 0.4	-17 ± 1

The formation and dissociation rate constant of the ERp57-CRT complex, in absence or presence of vancomycin, as a function of temperature were analyzed using the transition-state theory according to the Eyring equation to obtain the activation enthalpy ($\Delta H^{\ddagger}$) and entropy ($T\Delta S^{\ddagger}$) (Figure S7 and S8, Supplementary data). Table 2 summarizes the results obtained.

3.4. Binding of ERp57 to immobilized vancomycin

To better study the interaction of vancomycin with ERp57 and its influence on the formation of ERp57-CRT complex, vancomycin was covalently immobilized on chip surface and tested for ERp57 binding.

Fig. 4A shows the sensorgrams obtained when solutions of ERp57 were passed over a mixed SAM to which vancomycin had been immobilized. The binding observed in all cases was almost reversible. The equilibrium binding as well as the kinetic of association and dissociation of the protein onto the surface were evaluated with the Scatchard analysis [28]. The value of k_{off} was 0.0165 s^{-1}. The on-rate constant, k_{on}, was determined by calculating initial values of k_s, plotting those values of k_s against the concentration of ERp57 and using the value of the slope of the k_s versus [ERp57], as a measure of k_{on} (see Supplementary Information for more details). The values of k_{on} and K_D were 5000 M^{-1} s^{-1} and 3.3 10^{-6} M, respectively.

Competitive binding was performed using an ERp57 solution containing free vancomycin. Fig. 4B shows the sensorgrams obtained when solutions of ERp57 containing soluble vancomycin were passed over a mixed SAM to which vancomycin had been covalently immobilized. The presence of vancomycin in solution reduced the concentration of free ERp57. The initial rate of binding of ERp57 to the modified surface decreased with the increasing of the concentration of vancomycin in solution. When the concentration of vancomycin was higher than 45 μM, binding of ERp57 to the surface was completely inhibited. The value of K_D for the interaction between ERp57 and vancomycin was based on the decrease in the initial rate of binding of ERp57 to the immobilized vancomycin in presence of vancomycin as competitive inhibitor. We estimate $K_D = 4.0 \cdot 10^{-6}$ M.

As above reported for the ERp57-CRT interaction, the obtained values of K_C and K_S for the ERp57-vancomycin interaction are not significantly different, demonstrating again that the affinity of vancomycin for ERp57 at the sensor surface is identical to that in solution and K_C is not influenced by the sensor environment.

3.5. Effect of vancomycin on ERp57-CRT interaction on the cell surface

The effect of vancomycin on the ERp57-CRT interaction was further tested in vivo. ERp57 and CRT have been recently detected on the cell surface by different studies [6,14,15]. We also found ERp57 in this location in HeLa cells (data not shown).

In order to evaluate the effect of vancomycin on the ERp57-CRT interaction and complex formation at the plasma membrane, we performed an immunofluorescence experiment with anti-ERp57 and anti-CRT specific antibodies on non-permeabilized HeLa cells. In this way only ERp57 and CRT located on the cell surface could be visualized. As shown in Fig. 5, the administration of increasing concentrations of vancomycin (100 nM, 500 nM, 1 μM) induced a marked decrease in the amount of CRT on the cell surface compared to the untreated cells (C, control). A densitometric analysis of the fluorescence intensity related to cell surface CRT has been performed. The increasing concentration of vancomycin induced a significant decrease in the CRT fluorescence intensity, as shown in a graph (Figure S9, Supplementary data). At the same time, a weaker effect was observed for cell surface ERp57.

An immunofluorescence experiment was also conducted on permeabilized HeLa cells, so as to visualize the possible effect of vancomycin on ERp57 and CRT located in the intracellular compartments. Since it has been described that vancomycin has difficulties to diffuse through the plasma membrane [29], no

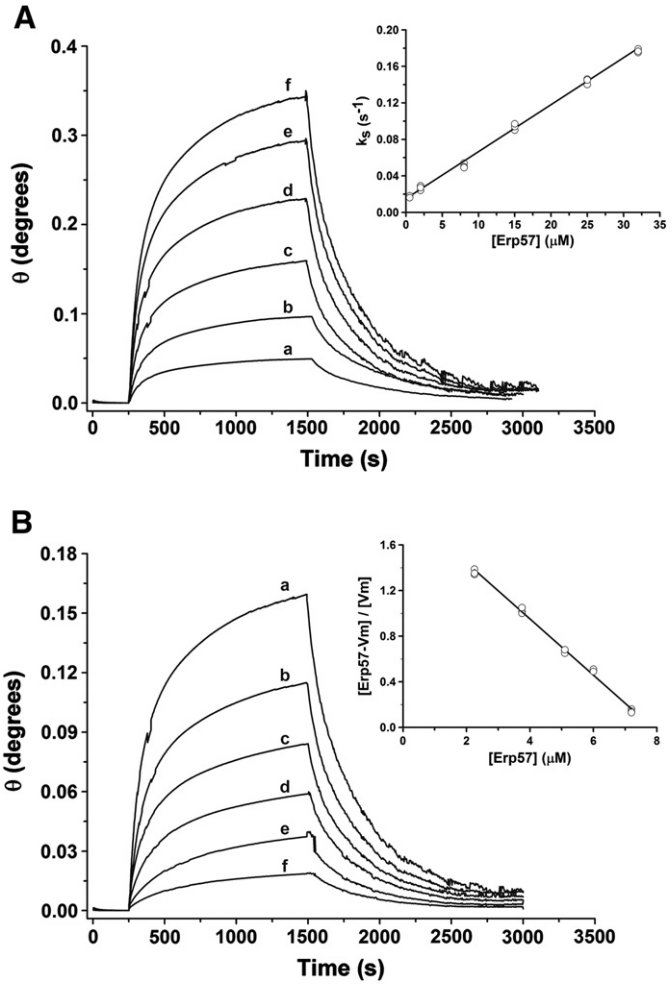

Fig. 4. (A) Sensorgrams corresponding to ERp57 binding to vancomycin immobilized on a mixed SAM. The concentrations of ERp57 in 10 mM Tris–HCl, pH 8.0, are (μM): 0.5 (a), 2.0 (b), 8.0 (c), 15.0 (d), 25.0 (e) and 32.0 (f). Inset: plot of k_s versus [ERp57] used to determine k_{on}. (B) Sensorgrams obtained by passing a solution of ERp57 (8.0 μM) containing different concentrations of vancomycin (0.0 (a), 1.5 (b), 3.2 (c), 7.5 (d), 13.0 (e) and 45.0 (f) μM) as competitive inhibitor over a mixed SAM to which vancomycin has been immobilized. Inset: Scatchard plot used for the determination of K_D for the interaction of ERp57 with vancomycin in solution.

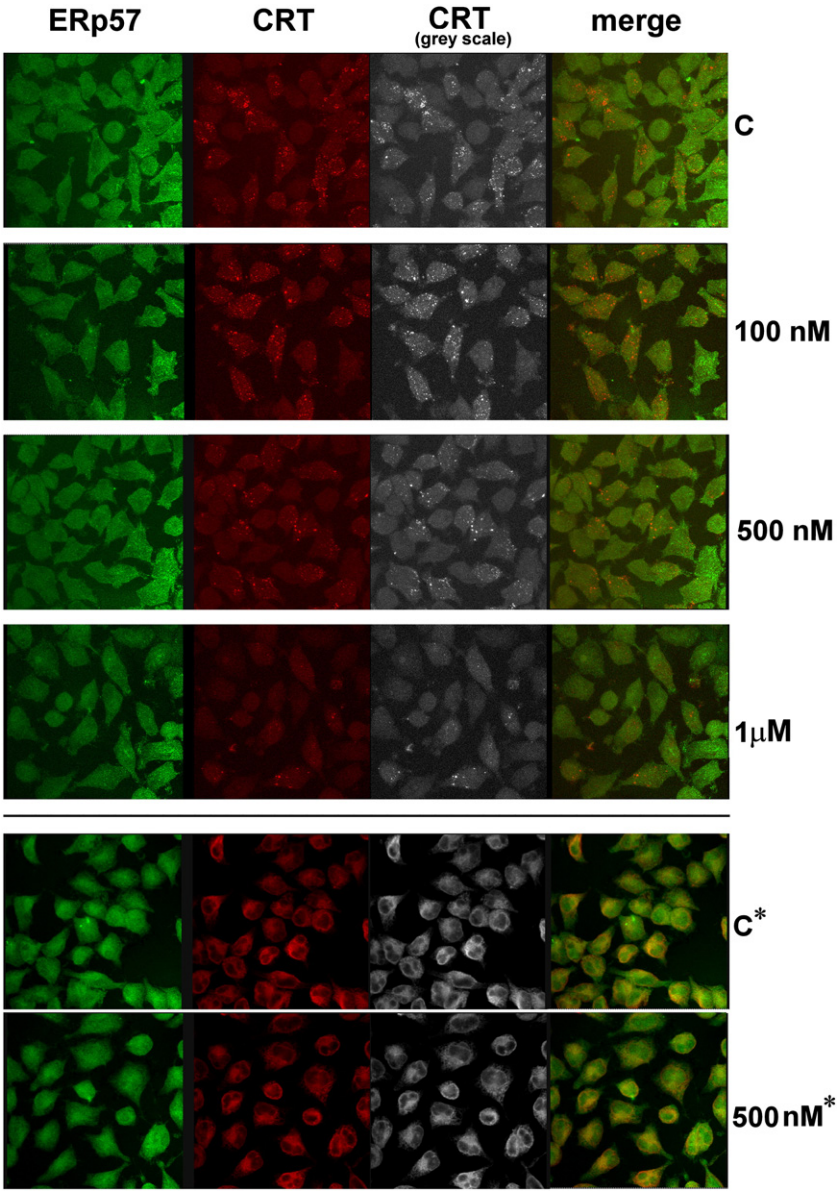

Fig. 5. Cell membrane surface distribution of ERp57 and CRT on HeLa cells. Cell were treated with different concentration of vancomycin (C as control,100 nM, 500 nM, 1 μM) and subjected to immunostaining with (C* and 500 nM*) and without (C, 100 nM, 500 nM, 1 μM) permeabilization step with 0,1% Triton X-100. Cells were immunostained with anti-CRT (rabbit serum) or anti-ER57 (mouse monoclonal antibody) followed by washing in PBS and incubation for 1 h with TRITC-conjugate goat-anti-rabbit-IgG or FITC-conjugated anti-mouse-IgG in the dark. Coverslips were mounted in Vectashield and analyzed by a Leica DM-IRE2 confocal microscope using a 40× objective. (See Figure S9, Supplementary data, for the densitometric analysis).

significant change in the two proteins could be detected inside the cells.

4. Discussion and conclusions

The ERp57-CRT interaction was analyzed by SPR obtaining a K_D of 2.6 μM, which is consistent with the values previously reported [18,19]. Taking into account the kinetic information contained in the SPR curves, it is possible to hypothesize a two-step binding model, where ERp57 could undergo a conformational change to stabilize its interaction with calreticulin.

Considering the various, well known, interactors of ERp57, i.e. vasopressin [30], the angiotensin-II receptors [31], the lectin calreticulin, the transcription factor STAT3 [32,33], as well as vitamin D_3 [6], it could be taken into account a plasticity in the ERp57 structure that allows specific changes or conformational "fine-tuning" to handle all the different interactions. Moreover, the difficulty to obtain structural information of the protein alone by crystallographic studies underlines the peculiarity of the protein and the presence of dynamic and/or disordered structures which are responsible for the great adaptability of ERp57 in the different interactions. Better information was obtained when ERp57 structure was analyzed in presence of a ligand. Recently, a structural study of tapasin-ERp57 complex has been published [16], whereas the interaction of calreticulin P domain with ERp57 b-b′ domains has been previously analyzed [34]. The two-step binding model here proposed provides kinetic and thermodynamic evidences for an adaptive ERp57 conformational change and shows the first binding step contributing the most on the overall binding free energy.

It has been previously reported that vancomycin can bind reversibly to ERp57 and inhibits the reductase and the DNA-binding activities of the protein [22]. In this report we confirmed the binding capability of vancomycin to ERp57 and provided evidences on the role of vancomycin as inhibitor of the ERp57/CRT interaction. The

effect of vancomycin on the ERp57-CRT interaction was further investigated by kinetic and thermodynamic analysis. Our results, obtained in absence and presence of vancomycin, showed a difference in the binding constants caused by the antibiotic (see Tables 1 and 2). The energy transitions in the binding process are also schematically represented in Figure S6 (Supplementary data). The vancomycin seems to interfere in the ERp57-CRT interaction, particularly in the first step of the hypothesized model (see in Table 1 the K_{a1} decreases of two orders of magnitude in the presence of antibiotic). Moreover, the K_D value for ERp57-CRT binding increases by about one order of magnitude. This variation leads to the hypothesis that the interaction between ERp57 and vancomycin could hinder the conformational changes necessary to stabilize ERp57-CRT interaction, as highlighted in the two-step model assumed for ERp57-CRT binding. This finding was further confirmed from the thermodynamic analysis of the binding process.

The free energy change is the result of both enthalpy and entropy changes. The first one can be attributed to the breaking (positive enthalpy) and forming (negative enthalpy) of bonds, including hydrogen bonds and van der Waals interactions. Entropy variation, instead, can be attributed to changes in the degrees of freedom of a system, sometimes described as randomness [35]. This includes change in the conformational, rotational and translational degrees of freedom of all the components of the system, as well as solvent effects, such as the influence of hydrophobicity. Another contribution to the thermodynamics of protein–protein interaction is the release of water molecules from the interface upon binding [36,37]. The desolvatation of the surface of the protein and transfer of the water molecules to the bulk upon binding with the ligand, results in an enthalpy cost. Furthermore, comprehensive theoretical analyses indicate that the stability of ion-pair formation in proteins strongly depends upon the local dielectric constant [38]. When electrostatic contacts are the major source of protein stability, theoretical considerations and experiments demonstrate that the molecular recognition is driven by a favorable desolvatation entropy term and opposed by enthalpy [39].

Analyzing the ERp57-CRT binding, both in the presence and absence of vancomycin, a negative enthalpy for the formation of the transition state and a negative entropy contribution to the free energy were observed. However, the extent of the underlying enthalpy and entropy contributions was quite different, suggesting important differences in the binding mode for ERp57 and CRT in the presence or absence of vancomycin. The activation parameters for the transition state indicate that the ERp57-CRT interaction in the absence of vancomycin has a higher entropy cost than in the presence of vancomycin. Furthermore, the equilibrium parameters indicate a higher enthalpy price for CRT binding to ERp57 in the presence of vancomycin. A likely explanation for the higher enthalpy cost of the ERp57-CRT interaction is the restricted conformational freedom of ERp57 due to the binding with vancomycin.

Our data indicate a similar K_D value for the ERp57 interaction with calreticulin and vancomycin. This allows us to hypothesize that vancomycin may hinder the interaction between ERp57 and calreticulin but cannot inhibit it at higher concentration of calreticulin. Furthermore, an inhibition mechanism can be assumed where the interaction sites of vancomycin and calreticulin are overlapping and therefore the interaction with the antibiotic could interfere with the ERp57 conformational changes that stabilize the interaction between the two proteins. In this view, the hydrophobic b′ domain of ERp57 would make an excellent candidate as vancomycin interaction site. A molecular modeling study and the use of specific mutants could help to confirm such hypothesis.

Preliminary studies on cell cultures have been conducted to assess the effect of vancomycin administration on the interaction between ERp57 and calreticulin in vivo. We carried out an analysis by immunofluorescence confocal microscopy on HeLa cells, focusing on the ERp57-CRT interaction at the level of the plasma membrane surface. It has been reported in the literature the presence of ERp57 and calreticulin on the cell surface linked to an immunogenic apoptosis mechanism [15]. Our results showed a significant decrease in the amount of calreticulin on the plasma membrane in response to increased concentrations of vancomycin, although we were unable to show a similar effect for ERp57. Since calreticulin requires the interaction with ERp57 for its translocation to the plasma membrane [14], the observed decrease in the exposed lectin could be justified by the inhibitory effect of vancomycin on the ERp57-CRT interaction.

The experimental model presented in this report can be used for the detection of other specific inhibitors/interactors of ERp57, which could be useful to understand the biological pathways where the protein is involved and to modulate its activity.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bpc.2011.09.003.

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