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The Inhibition Mechanism of Guanidine Hydrochloride on the Catalytic Activity of Recombinant Human Protein Disulfide Isomerase

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Initial velocity enzyme kinetics was used to study the inhibition mechanism of guanidine hydrochloride (Gdm.Cl) on catalytic activity of recombinant human protein disulfide isomerase (rhPDI) in protein folding. Reduced C125A recombinant human interleukin 2 (C125A rhIL-2), the substrate, was dissolved in 8 M Gdm.Cl before it was diluted into the folding buffer to initiate the folding reactions. The final Gdm.Cl concentrations in the folding buffer were fixed at 0.2 M, 0.4 M, 0.6 M and 0.8 M. The reduced and native C125A rhIL-2 were resolved by reversed phase-high performance liquid chromatography (RP-HPLC). The simultaneous nonlinear fitting of the initial velocities of the native C125A rhIL-2 formation *vs* the reduced C125A rhIL-2 concentrations in the presence of different Gdm.Cl concentrations shows that the inhibition mechanism of Gdm.Cl on the catalytic activities of rhPDI is a mixed-type noncompetitive nonlinear inhibition.

Keywords: Protein disulfide isomerase; Guanidine hydrochloride; Enzyme inhibition; Protein folding; Interleukin 2

Abbreviations: C125A rhIL-2, C125A recombinant human interleukin 2; Gdm.Cl, guanidine hydrochloride; GSH, reduced glutathione; GSSG, oxidized glutathione; PDI, protein disulfide isomerase; rhPDI, recombinant human protein disulfide isomerase; RP-HPLC, reversed phased high performance liquid chromatography

INTRODUCTION

High level expression of eukaryotic protein in *Escherichia coli* results in the formation of inclusion bodies in the cell cytoplasm or periplasm.[1] The protein in the inclusion body is misfolded and has to be folded to its native structure to attain its biological activity. The folding of recombinant protein from inclusion bodies is typically accomplished by dissolving protein from inclusion in a strong chaotrope (guanidine hydrochloride (Gdm.Cl) or urea). A reducing reagent is added to reduce the protein disulfide bonds. The folding reaction is initiated by adding denatured/reduced protein in a denaturant into the folding buffer to allow the polypeptide to fold to its native structure.[2,3] The conventional protein-folding protocols are often not efficient for the renaturation of hydrophobic, disulfide-bond containing proteins because of the slow chemical conversion of thiols to disulfides during the protein folding and the tendency of unfolded protein to aggregate.[4]

A promising alternative of efficient renaturation of recombinant proteins is to utilize a folding system that closely mimics native protein production *in vivo*. Protein Disulfide Isomerase (PDI) is an enzyme that catalyzes the renaturation of disulfide-containing proteins.[5–11] Because disulfide exchange is usually the rate-limiting reaction in protein folding, PDI can be exploited to more efficiently fold therapeutic proteins *in vitro*.[5,12,13] Earlier results in our laboratory found that recombinant human PDI (rhPDI) enhances the *in vitro* folding of a model protein, C125A recombinant human interleukin 2 (C125A rhIL-2).[14] IL-2 is a key cytokine involved in the activation of T lymphocytes. It plays a crucial role early in the immune response, and is used for cancer therapy and the treatment of both immunodeficient

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and autoimmune diseases.[15] IL-2 contains structural attributes that make it a good model to study PDI-assisted folding. Native human IL-2 contains a free thiol group at cysteine 125 and a disulfide bond between cysteines at positions 58 and 105. C125A rhIL-2 is a mutant IL-2 by substitution of alanine for cysteine at position 125 (C125A rhIL-2), thus eliminating the possibility of incorrect disulfide isomer formation. C125A rhIL-2 serves as an excellent model protein to explore the PDI-assisted protein folding: the high level expression of human recombinant C125A rhIL-2 in *E. coli* results in the formation of insoluble misfolded inclusion bodies. In addition, the folding of reduced/denatured C125A rhIL-2 is limited by the relatively slow chemical conversion of thiols to disulfide bonds. Extensive aggregates of C125A rhIL-2 are formed during folding due to poor solubility at the neutral pH range needed for thiol oxidation.[16,17]

The efficiency of the rhPDI-assisted C125A rhIL-2 folding depends on rhPDI concentrations, GSH:GSSG (reduced glutathione:oxidized glutathione) ratio and temperature. We observed an unusual kinetic behavior during the rhPDI-assisted C125A rhIL-2 folding. The formation of native C125A rhIL-2 linearly increased with time in the beginning of the folding reaction, then slowed down.[14] Because the protocol of rhPDI-assisted C125A rhIL-2 folding, in the same way as for most folding protocols has to employ Gdm.Cl, a denaturant to dissolve unfolded protein, we suspect that residual Gdm.Cl in the folding mixture inactivated rhPDI which resulted in the decreased folding rate.

The effect of Gdm.Cl on the conformation and activity of PDI had been previously reported.[18,19] Gdm.Cl denatured PDI and deactivated its catalytic activity. However, the inhibition mechanism of Gdm.Cl on the catalytic activity of PDI is not clear. Hawkins and Freedman[18] tested the isomerase activity of PDI by reactivation of "scrambled" RNase in the presence of Gdm.Cl with different concentrations. It was found that PDI was very sensitive to inactivation by denaturants; 50% inactivation was observed in the presence of 0.2 M Gdm.Cl with complete inactivation in 1 M Gdm.Cl. Morjana and Gilbert *et al.*[19] suggested that, in the presence of low concentrations of Gdm.Cl, the denaturation of PDI was reversible and biphasic. It was found that PDI denatured by 6 M Gdm.Cl regained >90% of its original activity during glutathione-dependent reduction of insulin after insulin was diluted from 6 M Gdm.Cl to 0.5 M Gdm.Cl.[19] The objectives of this study were to determine whether deactivation of rhPDI by Gdm.Cl is responsible for the decelerated velocity of rhPDI-assisted C125A rhIL-2 folding, and to understand the inhibition mechanism of Gdm.Cl on the catalytic activity of rhPDI.

MATERIALS AND METHODS

rhPDI and C125A rhIL-2 were expressed, purified and characterized according to the previous report.[14] Briefly, rhPDI was expressed as a glutathione S-transferase fusion protein in *E. coli* cells. After the cells were harvested and lysed, the rhPDI-fusion proteins in the supernatant were purified by affinity chromatography. rhPDI was cleaved from GST by thrombin. C125A rhIL-2 inclusion bodies were harvested *from E. coli* carrying plasmid C125A rhIL-2 cDNA. After the C125A rhIL-2 inclusion bodies were isolated from cells, inclusion bodies were purified by multiple washing cycles with 5% triethanolamine-HCl and 4 M urea. The purity of rhPDI and C125A rhIL-2 was >95% as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, reversed phase high performance liquid chromatography (RP-HPLC) and Matrix-Assisted Laser Desorption Ionization-Time of Flight mass spectrometry. All other reagents were analytical grade, and were used as purchased without further purification. All experiments were performed at 25°C unless reported otherwise.

Denaturation/Folding and Quantitation of C125A rhIL-2

C125A rhIL-2 inclusion bodies were dissolved in 8 M Gdm.Cl, 350 mM Tris–HCl (pH = 8.0), 20 mM EDTA. Reduced glutathione (GSH) was added to reduce the intramolecular disulfide bonds of C125A rhIL-2. Folding of C125A rhIL-2 was initiated by adding denatured/reduced C125A rhIL-2 into the folding buffer. At various times, an aliquot of 100 μl reaction mixture was quenched with 5 μl of 2 M HCl and kept at 4°C until HPLC analysis. Each reaction was performed in triplicate. RP-HPLC was used to resolve denatured/reduced C125A rhIL-2 from native C125A rhIL-2. A Vydac reversed-phase C-4 column (4.6 × 250 mm, 5-μm resin) with a guard column was used for all analytical measurements. The RP-HPLC analysis was performed with a linear gradient elution that consisted of 5% acetonitrile in water and 0.05% trifluroacetic acid as mobile phase A, and 5% water in acetonitrile and 0.04% trifluroacetic acid as mobile phase B. The UV detection wavelength was 215 nm. The injection volume was 50 μL. The gradient with 0.7 ml/min flow rate started at 58% B, increased to 70% B in 11 min and decreased to 58% B in 1 min. The column was allowed to equilibrate for 8 min before the next injection.

Initial Velocity Determination of Native C125A rhIL-2 Formation

The linear portion of the progress curves in rhPDI-assisted C125A rhIL-2 folding was taken to be

the initial velocity of the folding reaction. The folding reactions were initiated by addition of 19 volumes of folding buffer to 1 volume of denatured/reduced C125A rhIL-2. The folding reactions were quenched at 60 s. To determine the optimal rhPDI concentrations for the initial-velocity analysis, the folding reactions were initiated in the same way as for the initial-rate determination, except that the final folding mixture contained 0.35, 0.7, 1.4, 2.8 and 5.6 μM rhPDI and the folding reactions were quenched at 30 s.

The Inhibition Mechanism of Gdm.Cl on the Catalytic Activity of rhPDI

The effect of pre-incubation of rhPDI with Gdm.Cl on the catalytic activity of rhPDI was assessed by the following method. rhPDI was incubated with Gdm.Cl of various concentrations for 2, 5 and 10 min, respectively before it was added to the folding buffers to start the folding reactions. The initial velocities of the native C125A rhIL-2 formation with different pre-incubation times *vs* Gdm.Cl concentrations were plotted.

Initial velocity kinetics of rhPDI-assisted C125A rhIL-2 folding at different Gdm.Cl concentrations were performed as following. C125A rhIL-2 was reduced and denatured in 8 M Gdm.Cl and 400 mM GSH. Reduced C125A rhIL-2 concentrations were 560 μM. The reduced C125A rhIL-2 was diluted 1, 2, 3 and 4 fold with 8 M Gdm.Cl to give four sets of reduced C125A rhIL-2 solutions with the C125A rhIL-2 and GSH concentrations being 560 μM/400 mM, 280 μM/200 mM, 210 μM/150 mM and 140 μM/100 mM, respectively. The solutions of reduced C125A rhIL-2 were step-diluted with 8 M Gdm.Cl/400 mM GSH, 8 M Gdm.Cl/200 mM GSH, 8 M Gdm.Cl/150 mM GSH and 8 M Gdm.Cl/100 mM GSH, respectively, to give various C125A rhIL-2 concentrations. The folding reactions were initiated by diluting reduced C125A rhIL-2 into the folding buffers. The dilution factors were 40, 20, 15 and 10 fold for the four sets of reduced C125A rhIL-2 solutions, to give 0.2 M, 0.4 M, 0.6 M and 0.8 M Gdm.Cl in the final folding buffer. The GSH concentrations were 10 mM after dilution. Other components of the folding buffer included 0.75 mM GSSG, 100 mM Tris–HCl (pH = 7.75), 2 mM EDTA, 5.6 μM rhPDI and various concentrations of reduced C125A rhIL-2. The initial folding velocity was defined as the concentrations (μM) of native C125A rhIL-2 formed per min. The initial folding velocities in the presence of rhPDI were calculated after subtracting the velocities from that of the control folding reactions, which used the same concentrations of BSA instead of rhPDI in the folding buffer.

Data Analysis

To determine the inhibition mechanism of Gdm.Cl on the catalytic activity of rhPDI, the reciprocals of initial velocities of the native C125A rhIL-2 formation *vs* the reciprocals of reduced/unfolded C125A rhIL-2 concentrations at four different Gdm.Cl concentrations were plotted. The slopes and intercepts obtained from the double reciprocal plots were used to construct the secondary plots that determined the linearity of the inhibition mechanism. The double-reciprocal plots and secondary plots revealed the inhibition type of Gdm.Cl on rhPDI catalytic activity. The initial parameters that describe the catalytic activity of rhPDI on the C125A rhIL-2 were estimated from the fitting of the simple Michaelis-Menten equation. The initial inhibition constants of Gdm.Cl on catalytic activity of rhPDI were estimated from the secondary plots of intercept *vs* Gdm.Cl concentrations and slopes *vs* Gdm.Cl concentrations. After the inhibition type was determined, the kinetic model and equation that describe the inhibition mechanism of Gdm.Cl on the rhPDI were depicted. The initial velocities of native C125A rhIL-2 formation *vs* reduced C125A rhIL-2 concentrations were then simultaneously fitted to the nonlinear version of Michaelis-Menten equations describing the inhibition mechanism. The final parameters were obtained by simultaneous nonlinear fitting of curves of the initial folding velocities *vs* reduced C125A rhIL-2 concentrations to the Michaelis-Menten equations, using Winnonlin software (SCI, NC).

RESULTS AND DISCUSSION

Initial velocity analysis is commonly used to study enzyme inhibition kinetics. By determining the initial velocities of product formation or substrate depletion at different substrate and inhibitor concentrations, the inhibition mechanism of an inhibitor on an enzyme can be characterized by the kinetic constants, such as Michaelis-Menten constants K_m, V_{max}, the maximum reaction velocity and k_{cat} which represents maximum numbers of moles of substrate that are converted to product each minute, per mole of enzyme ($K_{cat} = V_{max}/[E]$). In the initial enzyme kinetic analysis, the enzyme and substrate concentrations and initial velocity determination are usually taken into the consideration in optimizing the analytical conditions.[20]

The initial time-course of enzyme-assisted reactions is usually linear, but the rate starts to decline at late stages of the reaction. The decrease in product formation with time can be attributed to several causes, such as enzyme inhibition, product inhibition, substrate depletion, etc. However, at the very

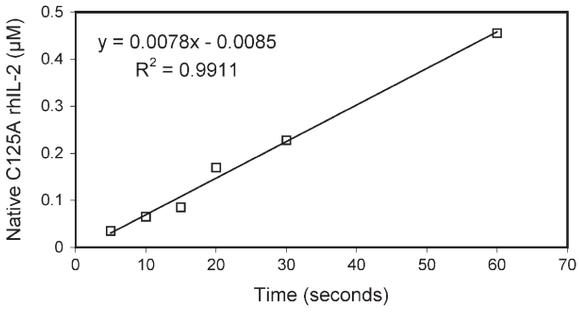


FIGURE 1 The initial velocity determination of rhPDI-assisted C125A rhIL-2 folding. The formation of native C125A rhIL-2 *vs* time in the first one minute of the folding reaction. The final folding buffer contained 1.4 μM C125A rhIL-2, 10 mM GSH, 0.75 mM GSSG, 100 mM Tris–HCl (pH = 7.75), 2 mM EDTA, 0.4 M Gdm.Cl and 5.6 μM rhPDI.

beginning of the reaction, these effects should not be significant if the initial linear portion of the folding reaction is studied.[20] Figure 1 shows that the formation of native C125A rhIL-2 linearly increases with time in the first minute of the folding reaction. The first 30 seconds of the folding reactions were chosen to reasonably approximate the initial rate of C125A rhIL-2 folding.

Theoretically, the initial velocities of enzyme-catalyzed reactions are proportional to the enzyme concentrations, where a graph of the initial velocities against enzyme concentrations is a straight line that passes through the origin. However, sometimes this simple relationship does not hold when enzyme concentrations are increased. Therefore, the linearity of the initial folding rates of C125A rhIL-2 as a function of rhPDI concentrations was examined. Figure 2 shows the relationship of the initial velocities of native C125A rhIL-2 formation *vs* rhPDI concentrations. The rate of native C125A rhIL-2 formation linearly increased with rhPDI concentrations from 0–5.6 μM. Based on this result,

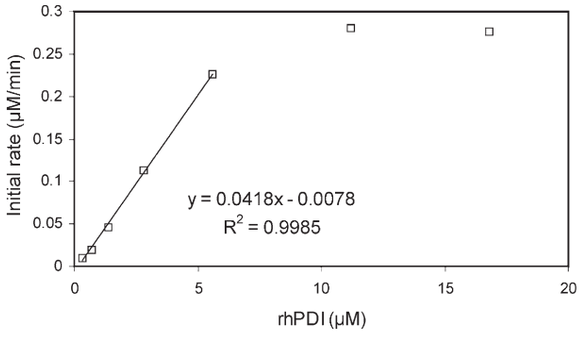


FIGURE 2 Optimal rhPDI concentrations for initial velocity analysis. The initial velocities of native C125A rhIL-2 formation *vs* rhPDI concentrations. The final folding buffer contains 1.4 μM C125A rhIL-2, 10 mM GSH, 0.75 mM GSSG, 100 mM Tris–HCl (pH = 7.75), 2 mM EDTA, 0.4 M Gdm.Cl and various rhPDI concentrations.

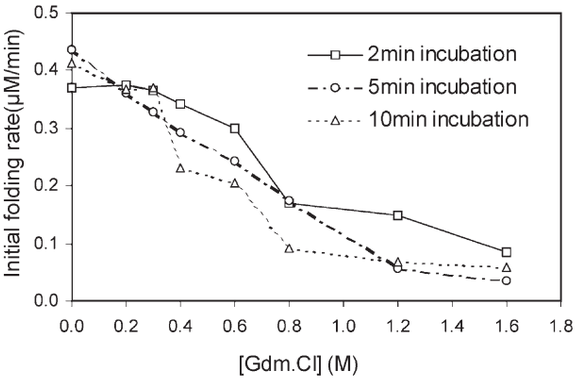


FIGURE 3 Effect of time of pre-incubating rhPDI with Gdm.Cl of different concentrations on its catalytic activity. The plots of initial folding velocities of the native C125A rhIL-2 formation *vs* Gdm.Cl concentrations when the pre-incubation times were 2 minutes (□), 5 minutes (○) and 10 minutes (△). The final folding buffer contained 1.4 μM C125A rhIL-2, 100 mM GSH, 0.75 mM GSSG, 100 mM Tris–HCl (pH = 7.75), 0.4 M Gdm.Cl, 2 mM EDTA and 5.6 μM rhPDI.

5.6 μM rhPDI or less was chosen to study the initial velocity kinetics of rhPDI-assisted C125A rhIL-2 folding.

A previous study shows that native C125A rhIL-2 formation linearly increased with time in the beginning of their folding reaction, and then slowed down.[14] Because we initiated the folding reaction by diluting reduced C125A rhIL-2 into the folding buffer containing both Gdm.Cl and rhPDI, we suggest that the inhibition of rhPDI catalytic activities by residual Gdm.Cl in the folding buffer was responsible for the decreased C125A rhIL-2 folding.

Pre-incubating rhPDI with Gdm.Cl of different concentrations demonstrates that Gdm.Cl inhibits rhPDI catalytic activity and the inhibition is Gdm.Cl concentration-dependent. Figure 3 shows that when rhPDI was pre-incubated with increasing concentrations of Gdm.Cl, the initial velocities of native C125A rhIL-2 formation decreased. Gdm.Cl is the most commonly used denaturant to dissolve recombinant proteins from inclusion bodies before protein solution is diluted into folding buffer to initiate the folding reaction. When the protein is very hydrophobic and water-insoluble, as is reduced denatured C125A rhIL-2, Gdm.Cl concentrations in the final folding buffer are usually maintained at minimal levels to keep the denatured proteins soluble because the soluble form of proteins is essential for subsequent folding. rhPDI catalyzes the renaturation of disulfide-containing proteins, which making it a promising agent in assisting renaturation of disulfide-containing recombinant proteins from insoluble misfolded inclusion bodies. However, it appears that rhPDI can be deactivated by denaturant Gdm.Cl in the final folding buffer. The potential utility of rhPDI in assisted-folding of recombinant therapeutic

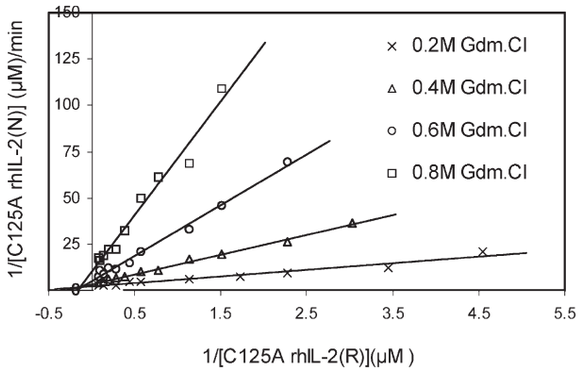


FIGURE 4 Plots of reciprocals of initial velocities *vs* reciprocals of C125A rhIL-2 (R) concentrations in the presence of Gdm.Cl at different concentrations. Gdm.Cl concentrations are 0.2 M (×), 0.4 M (△), 0.6 M (○), and 0.8 M (□). The solid lines are fitted curves of the reciprocals of the initial-rate *vs* reciprocals of C125A rhIL-2 (R)] (μM).

proteins can be severely compromised due to the potent inhibitory effects of residual Gdm.Cl in the folding buffer.

The Inhibition Mechanism of Gdm.Cl on rhPDI

The inhibition pattern exhibited by Gdm.Cl in a double reciprocal plot shows four straight lines that intersect off the y-axis as shown in Figure 4, which is consistent with a mixed-type noncompetitive inhibition mechanism. The secondary plots of slope *vs* Gdm.Cl concentrations and intercepts *vs* Gdm.Cl concentrations in Figures 5a and 5b are generated from the double reciprocal plots of Figure 4. The secondary plots show a linear relationship between the slopes and Gdm.Cl concentrations. However, there is an apparent deviation from linearity between y-intercepts and Gdm.Cl concentrations. The secondary plots suggest that the inhibition of Gdm.Cl on rhPDI is nonlinear.

Noncompetitive inhibition occurs when an inhibitor binds to the enzyme, and the conformation of either the substrate or the enzyme is distorted precluding catalysis. When an inhibitor adds to different enzyme forms, the inhibition is linear. However, when multiple inhibitors bind to a single enzyme form or to enzymes which are connected, replots of either slopes or intercepts, or both, against inhibitor concentrations may be nonlinear.[21] The mixed-type nonlinear noncompetitive inhibition of Gdm.Cl on rhPDI activity affects the intercepts, which shows a typical I-parabolic noncompetitive inhibition. Considering the inhibition mechanism of Gdm.Cl on rhPDI as in Scheme 1.

Equation 1 is used to describe the mixed-type nonlinear noncompetitive inhibition mechanism of Gdm.Cl on rhPDI,[21] where E and S are

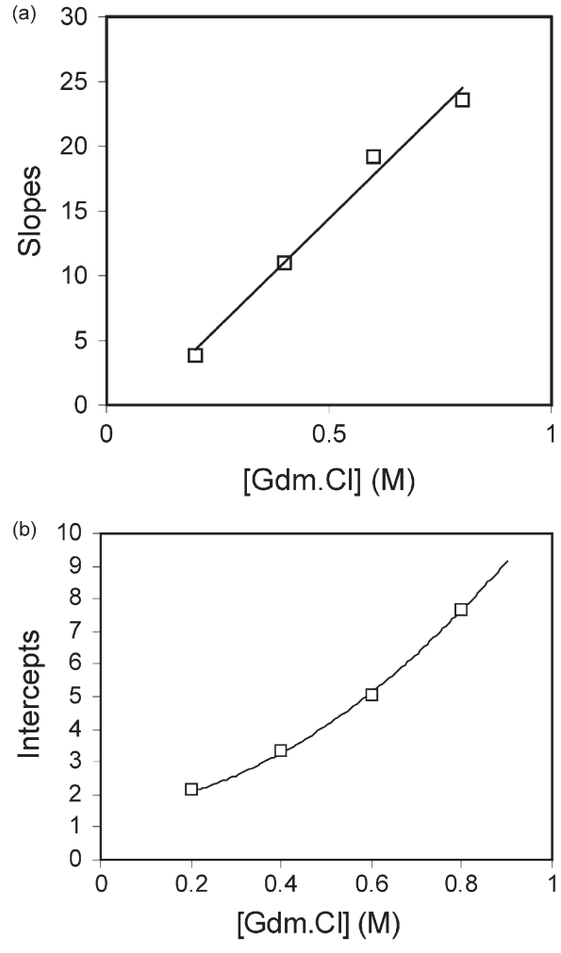
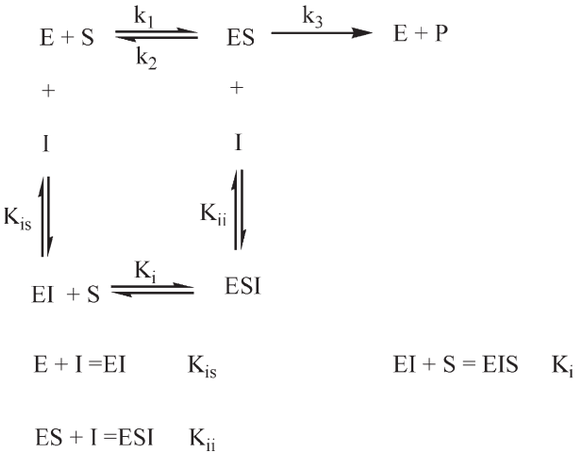


FIGURE 5 The secondary plots of slopes and intercepts *vs* Gdm.Cl concentrations from the double reciprocal plots. Figure 5a shows that plot of slope *vs* [Gdm.Cl] (M) is linear. Figure 5b shows that plot of intercepts *vs* [Gdm.Cl] (M) is nonlinear.



SCHEME 1

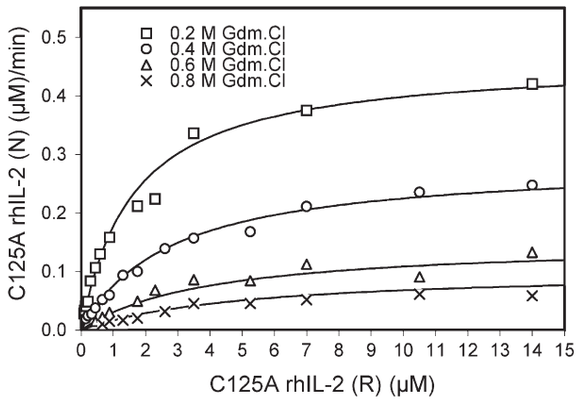


FIGURE 6 The simultaneous nonlinear fitting of the complex Michaelis-Menten equation of rhPDI-assisted C125A rhIL-2 folding in the presence of different Gdm.Cl concentrations. Gdm.Cl concentrations are (0.2 M (□), 0.4 M (○), 0.6 M (△), and 0.8 M (×)). The solid curves are the fitted curves of experimental data.

the concentrations of rhPDI and C125A rhIL-2, respectively. Gdm.Cl concentrations is represented as I. K_i, K_{ii} and K_{is} are the association constants of rhPDI and Gdm.Cl. V is the initial velocity of the native C125A rhIL-2 formation. V_{max} and K_m are the maximal velocity of the folding reaction and Michaelis-Menten constant, respectively.

$$\frac{1}{V} = \frac{1}{V_{\max}}\left(1 + \frac{[I]}{K_i} + \frac{[I]^2}{K_i \times K_{ii}}\right) + \frac{K_m}{V_{\max}} \times \left(1 + \frac{[I]}{K_{is}}\right) \times \frac{1}{[S]} \qquad (1)$$

The initial folding velocities *vs* substrate concentrations at four different Gdm.Cl concentrations were simultaneously fitted to the Michaelis-Menten equation (Equation 2) to further confirm the inhibition mechanism and to estimate the intrinsic constants k_3, K_m and the inhibition constants of Gdm.Cl on rhPDI, as shown in Figure 6. The parameters obtained from the fitting are shown in the Table I. The analysis of residuals between the actual curves and predicted curves and the parameter standard errors indicates a good fit of the model to the data sets with fair precision in the parameter estimates.[22]

$$V = \frac{k_3 \times E}{(1 + \frac{[I]}{K_i} + \frac{[I]^2}{K_i \times K_{ii}})} \times \frac{[S]}{K_m \times (1 + \frac{[I]}{K_{is}}) + [S]} \qquad (2)$$

In summary, the results presented in this paper suggest that inactivation of rhPDI by residual Gdm.Cl in the folding buffer is responsible for the decelerated velocity of rhPDI-assisted C125A rhIL-2 folding. The inhibition pattern exhibited by Gdm.Cl is via a mixed-type noncompetitive nonlinear inhibition mechanism. However, decreasing Gdm.Cl concentrations in the folding buffer may not positively affect the final folding yields because Gdm.Cl is an important reagent in maintaining the solubility of denatured proteins and the soluble form of denatured protein is essential for efficient protein folding. During the rhPDI-assisted folding, it appears that the residual Gdm.Cl concentrations need to be carefully optimized to maximize the protein folding efficiency.

TABLE I Kinetic constants obtained from the simultaneous nonlinear fitting of the complex Michaelis-Menten equation of rhPDI-assisted C125A rhIL-2 folding at different Gdm.Cl concentrations

Parameter	K_i (μM)	K_{ii} (μM)	K_{is} (μM)	k_3 (min^{-1})	K_m (μM)
Estimate	0.168	0.67	0.071	0.21	0.42
StdError	0.067	0.26	0.010	0.045	0.034

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