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Ligand Binding Analysis and Screening by Chemical Denaturation Shift

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Abstract

The identification of small molecule ligands is an important first step in drug development, especially drugs that target proteins with no intrinsic activity. Towards this goal, it is important to have access to technologies that are able to measure binding affinities for a large number of potential ligands in a fast and accurate way. Since ligand binding stabilizes the protein structure in a manner dependent on concentration and binding affinity, the magnitude of the protein stabilization effect elicited by binding can be used to identify and characterize ligands. For example, the shift in protein denaturation temperature (T_m shift) has become a popular approach to identify potential ligands. However, T_m shifts cannot be readily transformed into binding affinities and the ligand rank order obtained at denaturation temperatures (60°C or higher) does not necessarily coincide with the rank order at physiological temperature. An alternative approach is the use of chemical denaturation, which can be implemented at any temperature. Chemical denaturation shifts allow accurate determination of binding affinities with a surprisingly wide dynamic range (high micromolar to sub nanomolar) and in situations in which binding changes the cooperativity of the unfolding transition. In this paper we develop the basic analytical equations and provide several experimental examples.

Introduction

The linkage between conformational and binding equilibrium has been known for over sixty years thanks to the seminal work of Wyman [1,2]. The structural stability of a protein is determined by its Gibbs energy of stability, G , which is a function of temperature, chemical denaturants and other physical or chemical variables [3-7]. The temperature dependence of G is given by:

$$\Delta G = \Delta H_{T_r} - T \Delta S_{T_r} + \Delta C_p (T - T_r) - T \Delta C_p \ln \left(\frac{T}{T_r} \right) \quad (1)$$

where T_r is the reference or reporting temperature and H , S and C_p are the respective changes in enthalpy, entropy and heat capacity associated with the folding of the protein. In the presence of chemical denaturants like urea or guanidinium hydrochloride (GdnHCl), the

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Gibbs energy has been experimentally shown to follow a simple linear dependence with the denaturant concentration [5,8-12]:

$$\Delta G = \Delta G^o - mC \quad (2)$$

Where G^o is the Gibbs energy of stability of the protein at zero denaturant concentration; i.e. the stability of the protein at the same experimental temperature and solvent conditions except denaturant. C is the denaturant concentration and m is universally referred to as the m -value [8-11]. In protein stability studies it is customary to use the native state as the reference state [4], consequently a positive G indicates that the native state is more stable than the denatured state. The denaturation midpoint occurs at the temperature or at the denaturant concentration where $G = 0$. From equation 2 it is easy to conclude that the denaturant concentration, $C_{1/2}$, at the denaturation midpoint is equal to $C_{1/2} = G^o/m$.

The stability of a protein is also affected by the presence of ligand molecules. A ligand that binds to the native state stabilizes that conformation in a manner dependent on its binding affinity and concentration [2,13]:

$$\Delta G_L^o = \Delta G^o + RT \ln (1 + K_a [L]) \quad (3a)$$

Or, equivalently

$$\Delta G_L^o = \Delta G^o + RT \ln \left(1 + \frac{[L]}{K_d} \right) \quad (3b)$$

In the above equations ΔG_L^o is the Gibbs energy in the presence of ligand L , $[L]$ is the free ligand concentration and K_a and K_d , the ligand association and dissociation constants respectively. It is clear that the presence of a ligand will increase the Gibbs energy in a manner dependent on ligand concentration and affinity.

The effect of ligand binding on protein stability has been used in drug discovery to screen for potential ligands. The approach, however, has been limited primarily to temperature denaturation detected by fluorescence [14-17] or by differential scanning calorimetry [18,19]. In both cases, the observation of a shift in the denaturation temperature (T_m) of the protein to higher temperatures is indicative of binding. Potential ligands are usually ranked in terms of the magnitude of the shift in T_m , since estimation of binding affinities at room or physiological temperature requires knowledge of the changes in enthalpy and heat capacity for both protein denaturation and ligand binding. This is an impossible task in a screening situation because it assumes knowledge of the binding thermodynamics of yet unknown ligands. Also, for ligands with different signs and magnitudes of their binding enthalpies, the ligand rank order obtained at the denaturation temperature (usually around 60°C) may not coincide with the rank order at physiological temperature. Despite these drawbacks, the T_m shift approach has become very popular due primarily to its ease of implementation.

An alternative to the T_m shift approach is the chemical denaturation shift approach. Increases in protein stability in chemical denaturation due to substrate or ligand binding have been reported as early as 1980 and related to the binding affinity of ligands [20]. More recently, chemical denaturation has been successfully used to estimate the binding of ligands to FKBP-12 [21,22]. In this case, rather than an increase in T_m the approach measures the increase in the concentration of denaturant (e.g. urea or GuHCl) required to denature the protein in the presence of a ligand. Chemical denaturation curves, however, depend on two parameters, the Gibbs energy of protein stability and the m value which is proportional to the change in solvent exposure upon denaturation or the cooperativity of the transition [11].

As discussed in this paper, the chemical denaturation shift does provide sufficient information to estimate binding affinities but, until now, it has been difficult to implement. In the past, estimation of binding parameters from chemical denaturation curves assumed that the free ligand concentration could be approximated by the total ligand concentration, an approximation which is valid only if the ligand concentration is much higher than the protein concentration [21]. The use of this approximation precludes accurate characterization of high affinity ligands. Only recently, the total ligand transformation equation [19,23] has been incorporated in the analysis [22]. In this paper, we present the basic theory for the analysis of binding induced chemical denaturation shifts including the total ligand concentration transformation and allowing for m value differences between free and bound protein. We present experimental examples with three protein systems and ligands with binding affinities spanning six orders of magnitude. These examples illustrate the ability of the technique to measure binding affinities down to the picomolar level and ligand binding affinities for proteins that exhibit intrinsically disordered domains that become ordered (reflected in increases in m values) upon binding.

Theory

Gibbs Energy and Ligand Binding

Chemical denaturation in the presence of a ligand is described by the equation

$$\Delta G = \Delta G_L^o - m_L C \quad (4a)$$

$$\Delta G = \Delta G^o + RT \ln \left(1 + \frac{[L]}{K_D} \right) - m_L C \quad (4b)$$

which is obtained by combining equations 2 and 3. For completeness, the m value (m_L) is allowed to be different in the absence and in the presence of a ligand, a situation that can be expected if the ligand alters the cooperative behavior of the unfolding reaction [11]. Chemical denaturation experiments in the absence and in the presence of ligand yield ΔG^o and ΔG_L^o . In principle, the difference between the two ΔG values can be used to estimate the binding affinity:

$$\Delta \Delta G^o = \Delta G_L^o - \Delta G^o = RT \ln \left(1 + \frac{[L]}{K_D} \right) \quad (5)$$

Which after rearranging provides an equation for K_D :

$$K_D = \frac{[L]}{\left(\exp \left(\frac{\Delta \Delta G^o}{RT} \right) - 1 \right)} \quad (6)$$

Equation 6 is written in terms of the free ligand concentration which is unknown. In the past, the assumption has been made that $[L]$ can be approximated by the known total ligand concentration $[L_T]$ [21], an assumption valid when the ligand concentration is much higher than the protein concentration. In general, for a rigorous data analysis, all the equations need to be rewritten in terms of the known total ligand concentration.

Chemical Denaturation Shift

Chemical denaturation experiments yield three quantities ΔG^o , m value and $C_{1/2}$. Of the three quantities, $C_{1/2}$ is the one that can be determined with the smallest experimental error

[24,25] and therefore it has been the preferred quantity in data analysis. In the presence of a ligand, equation 4 allows derivation of the following equation for $C_{1/2}$:

$$C_{1/2} = C_{1/2}^o \frac{m}{m_L} + \frac{RT}{m_L} \ln \left(1 + \frac{[L]}{K_D} \right) \quad (7)$$

From which:

$$C_{1/2} - C_{1/2}^o \frac{m}{m_L} = \frac{RT}{m_L} \ln \left(1 + \frac{[L]}{K_D} \right) \quad (8a)$$

$$\Delta C'_{1/2} = \frac{RT}{m_L} \ln \left(1 + \frac{[L]}{K_D} \right) \quad (8b)$$

For those cases in which the m value is the same in the absence and in the presence of ligand, $\Delta C'_{1/2}$ is simply the difference of the $C_{1/2}$ values determined in the presence and absence of ligand:

$$\Delta C_{1/2} = C_{1/2} - C_{1/2}^o = \frac{RT \ln \left(1 + \frac{[L]}{K_D} \right)}{m} \quad (9)$$

This situation is very common. In fact, average m values are often used to compare the stability of proteins under different conditions [22,24].

In all the above equations, $[L]$ is the free ligand concentration. In the experiment the known quantity is the total ligand concentration, $[L_T]$, which is equal to the bound ligand concentration, $[L_B]$, plus the free ligand concentration ($[L_T] = [L] + [L_B]$). Only in those cases in which the protein concentration is much less than the total concentration of ligand then the free and total concentrations of ligand are similar. In general, the above equations need to be modified to account for the difference between total and free ligand concentration. This modification is standard and similar to the one used in isothermal titration calorimetry [19,23] and has been recently applied to the analysis of chemical denaturation [22]. The free ligand concentration is expressed in terms of the total ligand and protein concentrations in terms of the standard equation [19,23]:

$$[L] = \frac{-([P_T]/K_d - [L_T]/K_d + 1) + \sqrt{([P_T]/K_d - [L_T]/K_d + 1)^2 + 4[L_T]/K_d}}{2/K_d} \quad (10)$$

from which:

$$\Delta C'_{1/2} = \frac{RT}{m_L} \ln \left(1 + \frac{-([P_T]/K_d - [L_T]/K_d + 1) + \sqrt{([P_T]/K_d - [L_T]/K_d + 1)^2 + 4[L_T]/K_d}}{2} \right) \quad (11)$$

Equation 11 provides a rigorous mathematical relationship between the chemical denaturation shift, the m values and the protein and total ligand concentrations which are known. The only unknown parameter is the ligand dissociation constant K_d which can be calculated if the other parameters are known.

Figure 1 (left panel) shows the dependence of the chemical denaturation shift on the total concentration of ligand for a fixed protein concentration and different binding affinities. For

simplicity the m values have been assumed to be the same for the unliganded and liganded protein. This figure clearly illustrates the dynamic range of the technique and the ability to discriminate a wide range of binding affinities. Figure 1 (right panel) shows the effect of protein concentration on $C_{1/2}$ for a given binding affinity. This figure emphasizes the need to use the total ligand concentration correction when the protein concentration required for measurements is high. As a rule of thumb, differences between free and total ligand concentration become noticeable when the protein concentration is larger than 20-fold the K_d .

The Binding Affinity

Further algebraic manipulation of equation 11 allows derivation of an equation for K_d . For convenience we define

$$F \equiv \exp \left(\frac{m_L \Delta C'_{1/2}}{RT} \right) - 1 \quad (12)$$

And derive the following equation for K_d

$$K_d = \frac{[L_T] (1+F) - F [P]}{F (1+F)} \quad (13)$$

Equation 13 allows calculation of K_d in terms of the known quantities: total ligand concentration, protein concentration, the chemical denaturation shift and the m value. Figure 2 illustrates the dependence of K_d on $C_{1/2}$ for typical experimental conditions and demonstrates the level of resolution that can be achieved. For example, for a typical protein with m and $C_{1/2}$ values of 2000 kcal/mol/M and 4M, K_d values down to 1pM can be easily measured using a protein concentration of 1μM.

Experimental Tests

The experimental accuracy of the approach described above was tested by analyzing the binding of different inhibitors to three different protein systems with binding affinities ranging from 10μM to 60pM. The three proteins are human carbonic anhydrase (hCA-I), the HIV-1 protease and the envelope glycoprotein from HIV-1 (gp120). gp120 is known to possess intrinsically disordered domains that become structured upon binding [26]. In all cases discussed in this paper, changes in the intrinsic tryptophan fluorescence of the proteins were used to monitor protein denaturation. In general, the nature and magnitude of the observed fluorescence changes (wavelength shift, emission intensity, peak shape, etc) depend on the number of tryptophans and their specific environments in the native and denatured states (see for example [27] for a review). For thermodynamic analysis of protein denaturation the only requisite is that the native and denatured states exhibit a difference in any of the spectral properties (intensity, wavelength of maximum, etc). If a protein lacks tryptophans or no changes in intrinsic fluorescence are observed, chemical denaturation can also be followed by using extrinsic probes like ANS, sypro orange, Nile Red, etc [28].

Figure 3 shows three normalized urea denaturation curves for human carbonic anhydrase I (hCA-I) in the absence and in the presence of 5 and 10 μM of the inhibitor TFMSA. In the absence of inhibitor the denaturation midpoint of hCA-I occurs at $C_{1/2} = 4.2$ M urea, which increases to 5.53 M urea in the presence of 5 μM TFMSA and to 5.8 M urea at 10 μM TFMSA. The m value averages 1.47 kcal/mol/M urea and does not change in the presence of ligand within experimental error. Analysis of the data in terms of equation 13 yields K_d values of 163 and 184 nM for the two ligand concentrations, in excellent agreement with the

164 nM value determined by isothermal titration calorimetry with the same isozyme under identical conditions (ITC) (Table 1). Similar experiments were performed with the low affinity inhibitor ACTAZ. In this case, 200 μ M of the ligand was required to shift $C_{1/2}$ by 1.6 (data not shown). Analysis of the data yielded a K_d of 9.2 μ M also in excellent agreement with the value of 11.6 μ M determined by ITC under identical conditions. It should be pointed out that inhibitor binding to carbonic anhydrase is highly dependent on pH, buffer conditions and isozyme class [16,29].

In order to test the dynamic range of the chemical denaturation binding analysis, high affinity inhibitors of the HIV-1 protease were analyzed by GdnHCl denaturation shifts. Figure 4 shows the normalized denaturation curves for the HIV-1 protease in the absence and in the presence of 10 μ M of the inhibitor KNI-10769 and in the presence of 2.5 μ M of the inhibitor KNI-10006. It is immediately obvious that KNI-10006 produces a larger $C_{1/2}$ shift despite being assayed at a 4-fold lower concentration. Analysis of the data in terms of equation 13 yields K_d values of 21nM and 63pM for KNI-10769 and KNI-10006 respectively. These values are in excellent agreement with previously published values (11.8nM and 140pM) from this laboratory [30] obtained by ITC competition experiments under the same conditions.

Additional experiments were performed with the binding of the inhibitor NBD-556 to the HIV-1 envelope glycoprotein gp120 [31]. gp120 contains largely disordered domains that become structured upon binding of the inhibitor [26]. Figure 5 shows urea denaturation experiments of gp120 in the absence and in the presence of NBD-556 at 50 and 100 μ M. As expected, the curve becomes sharper and consequently is characterized by a larger m value, 2.43 kcal/mol/M-urea in the presence of the inhibitor versus 1.74 kcal/mol/M-urea in its absence. It has been shown before [11] that the m values are proportional to the magnitude of the change in solvent accessible surface area upon unfolding. Since NBD-556 binding triggers structuring in gp120 and a decrease in the solvent accessible surface area in the native state, the unfolding of the bound molecule is coupled to a larger change in solvent exposure and consequently a larger m -value. It has been shown before, that the structuring effect of NBD-556 is also reflected in the large unfavorable entropy associated with the binding of the inhibitor [26]. Analysis of the gp120 data in terms of equation 13 yields an average K_d value of 7.4 μ M, which is in excellent agreement with the 3.7 μ M value previously obtained by ITC under the same conditions[26].

Conclusions

Ligand induced perturbations in the chemical denaturation of proteins provide an accurate technique to estimate ligand binding affinity constants. The experiments presented in this paper as well as those presented before [21,22] demonstrate the wide dynamic range of the technique and the ability to reliably measure binding constants from the mid micromolar to mid picomolar range. In fact, the lower range is only limited by the solubility of the ligand. The upper range, on the other hand, is only limited by the magnitude of the fluorescence changes and the ability of the sensor to measure those changes. We estimate that picomolar or even tighter binding affinities can be measured in a dependable way. Standard chemical denaturants like urea or guanidinium hydrochloride can be used reliably to determine binding affinities. Only for strongly ionic ligands, guanidinium hydrochloride can be expected to influence binding affinity. Conventional techniques, like isothermal titration calorimetry require competition or displacement experiments in order to estimate binding affinities tighter than nanomolar. This technique seems ideally suited for screening a large number of compounds since measurements at a single ligand concentration are sufficient to obtain accurate estimates of binding affinity constants. Also, the chemical denaturation shift

approach provides an accurate way of determining binding affinities for very tight ligands that cannot be measured directly by other techniques.

Materials and Methods

Materials

Lyophilized human carbonic anhydrase isozyme I (hCA-I) was from Sigma-Aldrich (Cat. # C4396). Tethered HIV-1 protease containing a five amino acid linker[32] was expressed and purified as described elsewhere [33,34]. gp120 from the YU2 strain was expressed in human 293 F cells and purified as previously described in detail [35]. NBD-556 was kindly supplied by Dr. A. Smith, University of Pennsylvania, Philadelphia, PA. Trifluoromethane sulfonamide (TFMSA) and acetazolamide (ACTAZ) were from Sigma-Aldrich (Cat. # 638455 and A6011). KNI-10769 and KNI-10006 were kindly provided by Prof. Y. Kiso (Kyoto Pharmaceutical University, Kyoto, Japan). All the stock solutions of inhibitors were in 100 % DMSO at concentrations of 10 - 20 mM.

Chemical Denaturation Experiments

All denaturation experiments were carried out using an AVIA 2304 Automated Protein Denaturation System equipped with a fluorescence detector. This instrument automatically prepares and measures all samples starting from stock buffer, urea and protein solutions. In all cases, intrinsic tryptophan fluorescence was used to monitor denaturation. The excitation wavelength was 280 nm and scans of the emission intensity were recorded between 300 and 500 nm. Incubation times prior to fluorescence measurements were determined by measuring the fluorescence emission spectrum in the presence of denaturant until no change was observed. The experiments with hCA-I were carried out at pH 6.0 in a buffer composed of 10 mM sodium phosphate, 10 mM sodium succinate, and 10 mM L-histidine with 2 % DMSO. Since urea denaturation of hCA-I is slow, samples were incubated 18hrs before measurement. For each denaturation experiment, urea and buffer was dispensed into 24 wells with a linear increase in urea concentration from 0 to 9 M automatically generated by the instrument. The concentration of hCA-I in the wells was 0.5 μ M. Denaturation curves of hCA-I were carried out in duplicate without inhibitor and with either ACTAZ at 200 μ M or with TFMSA at 5 and 10 μ M, respectively. The denaturation experiments with HIV-1 protease were carried out using a linear concentration gradient of guanidine hydrochloride from 0 to 5.5M and measured immediately after preparation. The buffer was 10 mM sodium acetate, pH 5.0 with 2% DMSO. The final concentration of protease in the measuring well was 0.58 μ M. Denaturation experiments were performed in the absence of inhibitor and in presence of either KNI-10769 or KNI-10006 at concentrations of 10 and 2.5 μ M, respectively. The studies of gp120 were performed in PBS, pH 7.5 (Roche Diagnostics GmbH, Mannheim, Germany) with 2% DMSO and each denaturation curve was obtained using a linear gradient of urea as described above. The concentration of gp120 in the wells was 0.5 μ M and denaturation curves were carried out in the absence of inhibitor and in the presence of NBD-556 at 50 and 100 M, respectively.

Isothermal Titration Calorimetry

The binding of TFMSA and ACTAZ to hCA-I was measured by Isothermal Titration Calorimetry using a VP-ITC from MicroCal/GE Healthcare (Northampton, MA). hCA-I and the inhibitors were dissolved in buffer composed of 10 mM sodium phosphate, 10 mM sodium succinate, and 10 mM L-histidine set to pH 6.0 with 2 % DMSO. The titrations were performed at 25 °C by injecting 10 μ L aliquots of the inhibitor solutions into the calorimetric cell (volume ~ 1.4 mL) containing hCA-I at a concentration of 9.5 μ M. The concentrations of TFMSA and ACTAZ in the syringe were 100 and 200 μ M respectively. The heat evolved upon each injection of inhibitor was obtained from the integral of the

calorimetric signal. The heat associated with the binding reaction was obtained by subtracting the heat of dilution from the heat of reaction. The individual heats were plotted against the molar ratio, and the enthalpy change (ΔH) and association constant ($K_a = 1/K_d$) were obtained by nonlinear regression of the data. The binding of KNI-10769 and KNI-10006 to the HIV-1 protease and NBD-556 to gp120 has been measured before in this laboratory using the same buffer conditions as those used in the chemical denaturation experiments [26,30]

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Figure 1. Simulated dependence of $\Delta C_{1/2}$ on total ligand concentration for different values of the dissociation constant (K_d) and constant protein concentration (left panel) and different protein concentrations for a constant dissociation constant.

Figure 2.

The relationship between the dissociation constant (K_d) and the chemical denaturation shift ($\Delta C_{1/2}$) for different ligand concentrations at a constant protein concentration.

Figure 3.

Urea denaturation of hCA-I at pH 6.0 in the absence and presence of TFMSA. For each denaturation curve, hCA-I was dispensed into 24 wells to a final concentration of 0.5 μ M in a linear gradient of urea from 0 to 9 M. The buffer was composed of 10 mM concentrations of L-histidine, sodium succinate, and sodium phosphate with 2 % DMSO. The excitation wavelength was 280 nm. Data analysis was based on the difference between the intensities at 360 and 316 nm, which correspond to the fluorescence maxima of the denatured and native states, respectively (see inset). hCA-I alone (blue circles) denatures with a $C_{1/2}$ of 4.2 M, which increases to 5.6 M in the presence of 5 μ M TFMSA (red diamonds) and to 5.8 M when the concentration of TMSA is doubled to 10 μ M (green triangles). The wavelength shift that takes place during the denaturation is illustrated in the inset that shows fluorescence emission scans of hCA-I in 9 M urea (green) and in its native state in absence of urea (purple).

Figure 4.

Guanidine hydrochloride denaturation of HIV-1 protease in the absence (blue circles) and presence of the inhibitors KNI-10769 (red diamonds) and KNI-10006 (green triangles). The protein concentration in each set of 24 wells was 0.58 μ M in a linear gradient of guanidine hydrochloride from 0 to 5.5 M in 10 mM sodium acetate, pH 5.0 with 2% DMSO. The fluorescence intensities at 356 nm upon excitation at 280 nm were used to analyze the data. The presence of 10 μ M KNI-10769 (red diamonds) shifts $C_{1/2}$ from 1.8 to 2.8 M whereas KNI-10006 at 2.5 μ M shifts $C_{1/2}$ even further to 3.4 M. The inset shows fluorescence emission scans of the native (purple) and denatured (green) states of the protein.

Figure 5.

Urea denaturation of the HIV-1 envelope glycoprotein, gp120, in the absence and presence of the entry inhibitor NBD-556. The buffer was PBS, pH 7.5, with 2 % DMSO. The concentration of gp120 was 0.5 μM and the denaturation with urea was performed as described for hCA-I in Figure 3. The excitation wavelength was 280 nm and the difference in emission intensities at 354 and 346 nm was used to analyze the data. Binding of NBD-556 induces a large conformational structuring of gp120 which results in increased m-value for the inhibitor bound protein. This is observed in the shape of the curves; the denaturation curve for gp120 alone (blue circles) is characterized by an m value of 1.74 kcal/mol/M-urea and is notably less steep than the curves obtained in the presence of NBD-556 at 50 μM (red diamonds) and 100 μM (green triangles), which are characterized by an average m value of 2.43 kcal/mol/M-urea. The inset shows fluorescence emission scans of the native (purple) and denatured (green) states of the protein.

Table 1

Binding Thermodynamic Parameters Derived from Chemical Denaturation Experiments

Protein	Ligand	[Ligand] (μM)	Denatürant	m/mL	$C_{1/2}$ (M)	G_{binding} (kcal/mol)	K_d (nM)	K_d (nM)
hCA-I	TFMSA	5	Urea	1	1.36	−9.26(0.03)	163(9)	164(16) ¹
hCA-I	TFMSA	10	Urea	1	1.60	−9.20(0.08)	184(24)	
hCA-I	ACTAZ	200	Urea	1	1.26	−6.9(0.2)	9,200(1,600)	11,600(1,200) ¹
HIV-1 PR	KNI-10769	10	GdnHCl	1	1.03(0.07)	−10.5(0.1)	21(5)	11.8(0.66) ²
HIV-1 PR	KNI-10006	2.5	GdnHCl	1	1.64(0.11)	−13.9(0.3)	0.063(0.031)	0.14(0.04) ²
gpl20	NBD-556	50	Urea	0.72	0.55	−7.2(0.2)	6,400(2,400)	3,700(500) ³
gpl20	NBD-556	100	Urea	0.72	0.62	−6.9(0.3)	8,500(3,200)	

¹ Measured by ITC in this paper² Kawasaki et al [30]³ Sch n et al [26]