

# Measurement of barnase refolding rate constants under denaturing conditions

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## Abstract

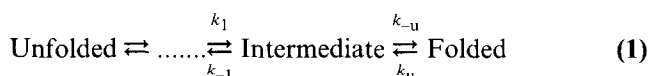
Calculation of equilibrium and kinetic constants between the different species that occur in the folding pathway of a protein usually depends on extrapolations from conditions where these quantities can be accurately measured. Direct measurements of the extrapolated constants would allow the checking of the validity of the extrapolations. We measure here refolding constants of barnase under unfolding conditions by displacing the folding equilibrium by complexing folded barnase with its polypeptide inhibitor barstar. Formation of the enzyme–inhibitor complex is very fast and virtually irreversible, and so refolding rate constants can be obtained. The results acquired confirm the extrapolations carried out in previous works.

**Key words:** Protein folding; Enzyme–inhibitor complex; Barnase; Barstar

## 1. Introduction

Working out the pathways followed by a polypeptide chain as it acquires its native conformation from its unfolded state requires the structural characterization of the initial, intermediate and final states, and the measurement of the rates of interconversion between those states. The structure of intermediates in the folding pathway of proteins have been mapped out by kinetics [1]. Intermediates can also be stabilized using covalent chemical reactions [2] or by protein engineering [3,4]. It is thus likely that the structures of many of the intermediates occurring in the folding process will be solved. The measurement of the rate constants of the events involved in folding is not always easy. Some of the reactions are too fast or too slow to be measured in water, and data obtained at high temperature or in the presence of denaturants must be extrapolated to standard conditions. We show here how direct measurements may be made on barnase.

The folding of the ribonuclease of *Bacillus amyloliquefaciens* (barnase) follows the scheme depicted in eq. (1)



where  $k_{-u}$  and  $k_u$  are the observed refolding and unfolding rate constants between the folded form and a major intermediate state which accumulates transiently before the major transition state for the reaction [1]. The rates of interconversion between the unfolded state and the intermediate,  $k_1$  and  $k_{-1}$ , are thought to be very fast. For wild-type barnase and most of its mutants,  $k_{-u} \approx 12 \text{ s}^{-1}$  in water [5] so this constant can be accurately measured with the currently available stopped-flow techniques. However,  $k_u$  in water ( $k_u^{\text{H}_2\text{O}}$ ) is estimated to be in the range of  $10^{-2}$  to  $10^{-4} \text{ s}^{-1}$  [6]. To calculate  $k_u^{\text{H}_2\text{O}}$ , unfolding data are collected in the presence of high concentrations of urea, where refolding is negligible and  $k_u$  has a measurable value. A linear extrapolation to 0 M urea has been used to calculate  $k_u^{\text{H}_2\text{O}}$  for staphylococcal nuclease [7] and this approach was initially followed for barnase [8]. More recently, a very precise analysis of the unfolding data of barnase suggests the use of a second-degree polynomial extrapolation rather than a linear one [6]. As a result, some of the new values differ significantly from the former calculated ones. It is desirable to use another approach to check the validity of such extrapolations.

Using equilibrium and unfolding data it is possible to determine the expected kinetic behaviour for a theoretical two-state folding process, with no kinetically significant intermediates present (eq. (2)) [1,9,10]



where  $k_F$  is the theoretical refolding rate constant ex-

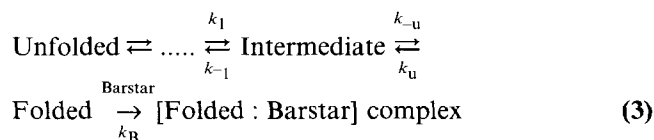
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Abbreviations:  $\Delta G_{A,B}$ , free energy of the state A relative to state B; U, unfolded state; I, intermediate state; F, folded state.

pected in this case, and it is evident that  $K_{UF} = k_u/k_F$  ( $K_{UF}$  being the equilibrium constant between the unfolded and the folded forms). In a two-state process,  $k_F$  coincides with the experimental  $k_{-u}$ . On the other hand, if the experimental data do not fit the two-state analysis, the existence of intermediates can be inferred [1].

Barnase is intracellularly synthesised together with a polypeptide inhibitor, barstar [11]. When barstar is added to barnase, eq. (1) has to be rewritten as follows (eq. (3))



The formation of the barnase–barstar complex may be considered irreversible at excess concentrations of barstar in the  $\mu\text{M}$  region. The addition of barstar to an equilibrium mixture of folded and unfolded barnase under these conditions will lead to the formation of the barnase–barstar complex in a multiphasic manner. There will be the rapid addition to the pre-existing concentration of folded state, followed by slower formation of more complex as the equilibrium is displaced by mass action. In many circumstances, the rate of formation of the subsequent complex follows a first-order rate law. Let the rate constant for this be  $k_{\text{obs}}$ . Since, as stated above, there is evidence that the  $U \rightleftharpoons I$  equilibrium is much faster than the  $I \rightleftharpoons F$  equilibrium, i.e.  $k_1 + k_{-1} \gg k_{-u}$ , then [12],

$$k_{\text{obs}} = k_{-u} \frac{K_{IU}}{1 + K_{IU}} \quad (4)$$

where  $K_{IU} = \frac{k_1}{k_{-1}} = \frac{[I]}{[U]}$ . Eq. (4) can also be rewritten as:

$$k_{\text{obs}} = \frac{k_F}{1 + K_{IU}} \quad (5)$$

Eq. (4) shows that by adding barstar to barnase in unfolding conditions for barnase it is possible, provided  $k_{-u}$  is known, to calculate directly the value of the equilibrium constant between the intermediate and the unfolded states,  $K_{IU}$ . It is important to note that this value would be obtained independently of other equilibrium or unfolding data, only  $k_{-u}$  being needed. An alternative approach is to use the extrapolated values of  $k_F$  and  $K_{IU}$  from earlier kinetic and equilibrium studies to calculate a theoretical value for  $k_{\text{obs}}$ , and then to compare the latter value with the experimental one. In any case, an agreement between the new experimental and the previous data would confirm the preceding approaches and, hence, the extrapolations involved from them. In this article we present a technique for measuring  $K_{IU}$  for some mutants of barnase by complexing them with barstar.

## 2. Experimental

### 2.1. Materials

The buffer used in the experiments was 50 mM MES (2-[N-mor-

pholino]ethanesulphonic acid), pH 6.3 (Sigma) (19.35 mM acid form and 30.65 mM sodium salt). Urea was enzyme grade from Bethesda Research Laboratories, MD, USA.

### 2.2. Expression and purification of barnase and barstar

Barnase mutants were expressed and purified as previously described [3,13]. Wild-type barstar was a generous gift of G. Schreiber.

### 2.3. Kinetic experiments on barnase

Barnase refolding and unfolding experiments in the absence of barstar were performed and analysed as described elsewhere [5,8].

### 2.4. Binding of barstar to barnase

In a typical experiment, 1  $\mu\text{M}$  barnase in 50 mM MES buffer, pH 6.3, and the appropriate urea concentration was mixed with an equal volume of a solution of 3  $\mu\text{M}$  barstar in the same buffer in an Applied Photophysics SF.17MV stopped-flow apparatus. Formation of the enzyme–inhibitor complex was followed from the emission of fluorescence above 315 nm or 335 nm upon excitation at 290 nm using a cut-off filter [14]. The monochromator slits were set to 2 nm bandpass. All measurements were carried out at 25°C. Data were analysed with Kaleidagraph (Abelbeck Software). The association constant for the formation of the barnase–barstar complex in non-denaturing conditions was calculated according to Schreiber and Fersht [14]. When barstar is added to barnase in denaturing conditions, two processes occur. While refolding of barnase generally produces an increase in fluorescence in the 315–335 nm region and a decrease above 335 nm, binding of barstar to barnase produces a general decrease in fluorescence. The overall process (refolding + binding) should proceed through a net increase in fluorescence, although this is a delicate balance. For some mutants, the best traces were obtained by measuring above 335 nm, where only the fluorescence decrease is monitored. The binding step is fast compared with the refolding step, and so it has no effect on the measurement of the latter, which is rate-limiting. Curves generally consist of two phases, the second one being very slow and associated with a component of *cis-trans* peptidyl-proline isomerization. The traces were fitted to eq. (6).

$$F(t) = A_1 \exp(-k_{\text{obs}} t) + A_2 \exp(-k_2 t) + C \quad (6)$$

where  $F(t)$  is the fluorescence at time  $t$ ;  $A_1$  and  $A_2$  are the amplitudes of the folding events,  $C$  is and offset,  $k_2$  is the rate of the second phase and  $k_{\text{obs}}$  is the rate constant of the major phase described in eq. (6). In some cases the second phase was better fitted as a drift (eq. (7))

$$F(t) = A_1 \exp(-k_{\text{obs}} t) + rt + C \quad (7)$$

$r$  being the drift.

## 3. Results and discussion

### 3.1. Conditions for the experiments

Suitable measurements of  $k_{\text{obs}}$  for the binding of barstar can be made only for some mutants and under some conditions. First, a sufficient excess of barstar is needed for the binding step to be faster than the  $I \rightarrow F$  reaction. Here, a three-fold molar excess of barstar over the total concentration of barnase (0.5  $\mu\text{M}$ ) was found to give the best results. Further, about 50% of barnase has to be initially unfolded to give a detectable fluorescence signal. The concentration of urea must be higher than or equal to that required for 50% denaturation. This limits the use to unstable mutants only, since barstar itself denatures above 4 M urea [14]. Finally, rates in the vicinity of 0.02  $\text{s}^{-1}$  are unreliable for reasons described below.

### 3.2. Affinity for barstar

The association constants for formation of the bar-

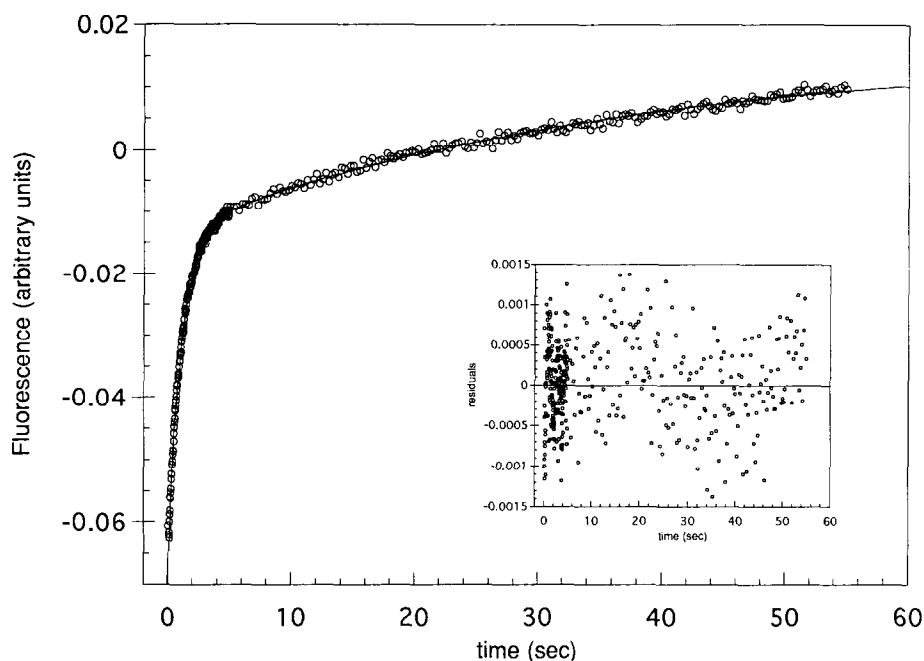


Fig. 1. Refolding of the I4A/I25V/I51V/Y78F barnase mutant monitored by binding to barstar. Barstar was added to barnase in the presence of 2.25 M urea, and the change in fluorescence above 335 nm was followed as described in section 2. The curve was fitted to a double exponential equation. The fit is shown in solid line. Inset: plot of the residuals after subtracting the experimental data from the fit.

nase–barstar complex in water for the different barnase mutants are all similar to that for wild-type protein [14] (data not shown). Values of  $k_B$  were measured for the I76T mutant in non-denaturing urea concentrations to check the effect of urea on the rate constant for binding to the folded state of barnase (Table 1). Results show that there is only a small effect of urea.

### 3.3. Analysis of the refolding curves

A typical trace is shown in Fig. 1. The binding of barstar to the initially folded molecules of barnase is too fast to be detected in the stopped-flow apparatus. The curves fit either to a double-exponential equation (eq. (6)) or a single-exponential plus a drift (eq. (7)), and the residuals show a random deviation from the fitted curve. In all cases, the lower rate constant is in the range of  $0.02 \text{ s}^{-1}$  independent of the concentration of urea. This rate constant is in the range previously found for the isomerization of *cis-trans* peptidyl-prolyl bonds.

The values of  $k_{\text{obs}}$  obtained for several mutants of

barnase are shown in Table 2. The mutants chosen for this study have different folding stabilities. Furthermore, the energy of the intermediate state relative to the unfolded state is essentially unaltered in some of the proteins (low  $\phi$  values, as described in Matouschek et al. [1] – see legend to Table 2), whereas it is destabilized in those with higher  $\phi$ . As depicted in this table, the theoretical  $k_{\text{obs}}$  values determined with eq. (5) using earlier calculated values of  $k_F$  and  $K_{\text{IU}}$  are very similar to the measured ones. This confirms the data that were acquired previously from extrapolation. There is an excellent agreement between the values of  $K_{\text{IU}}$  calculated from previous kinetic measurements [3] or with the new procedure, using eq. (4) (Table 2). It is noteworthy that, as expected, those mutants with  $\phi$  values closer to 0 exhibit the fastest refolding kinetics, in agreement with a high  $K_{\text{IU}}$  representative of accumulation of intermediate state, whereas those mutants with predicted low accumulation of intermediate (because of high  $\phi$  values and/or insufficient destabilization of the folded state [3]) present very low values of  $k_{\text{obs}}$ , often very difficult to distinguish from the slow phase of the experiment. This is the case of, for example, the D93N mutant. Although the folded state is very destabilized in this mutant (low urea midpoint of denaturation), the low  $k_{\text{obs}}$  predicts a low value of  $K_{\text{IU}}$  and subsequently a destabilization of the intermediate. Urea equilibrium denaturation monitored by fluorescence or far-UV CD display coincident denaturation curves (not shown), confirming a negligible accumulation of intermediate state in equilibrium [3].

Fig. 2 shows the experimental rate constants measured

Table 1  
Urea dependence of binding of barstar to barnase mutant I76T

| [Urea] (M) | $k_B^a \text{ (s}^{-1}\text{M}^{-1}) \times 10^{-8}$ |
|------------|--|
| 0.0        | $6.11 \pm 0.23$                                      |
| 1.0        | $5.72 \pm 0.16$                                      |
| 1.5        | $6.03 \pm 0.24$                                      |
| 2.0        | $4.71 \pm 0.11$                                      |

<sup>a</sup> $k_B$  is the second-order rate constant for association of barnase and barstar [14].

for the barnase mutants I4A/I51V, I4A/Y78F and I4A/I25V/I51V/Y78F with the barstar-binding procedure at different urea concentrations, together with the experimental rate constants of refolding and unfolding in the absence of barstar, the theoretical behaviour expected for a two-state transition ( $\log(k_u + k_F)$ ) and the extrapolation of  $k_F$  to concentrations of urea above the midpoint of denaturation. The values of  $k_F$  obtained with eq. (5) using the  $K_{IU}$  constants calculated in the absence of barstar [3] are also shown. These mutants were designed previously to accumulate the folding intermediate at concentrations of urea near the midpoint of denaturation [3]. The experimental rates of refolding thus differ from the two-state curves for these mutants. The calculated values of  $k_F$  all fit nicely to the extrapolation of  $\log k_F$  from the line for the two-state situation. This result demonstrates that  $k_F$  can be directly obtained in the presence of barstar. Moreover, this result indirectly demonstrates

that the extrapolation used to calculate  $k_u$  in water [6] is valid, since the latter value is needed to calculate  $k_F$ . Finally, as expected, the values of  $k_{obs}$  approach the  $k_F$  line as [urea] increases, consistently with a progressively lower  $K_{IU}$  which makes eq. (5) to approximate to  $k_{obs} = k_F$ .

#### 4. Concluding remarks

As described above, the barstar-binding procedure allows the measurement of refolding constants for barnase in unfolding conditions, thus testing experimentally the validity of extrapolation approaches and confirming properties of already known mutants. The system is limited to certain mutants and can be applied only under certain conditions, but the collected data can be interpreted in a very straightforward way. More precisely, it

Table 2  
Experimental rate and equilibrium constants for mutants of barnase

| Mutant                | [urea] <sub>1/2</sub><br>(M) <sup>a</sup> | $\phi_1^b$ | [urea]<br>(M) | $k_{obs}$ (s <sup>-1</sup> )<br>(experimental) <sup>c</sup> | $k_{obs}$ (s <sup>-1</sup> )<br>(expected) <sup>d</sup> | $K_{IU}$<br>(this study) <sup>e</sup> | $K_{IU}$<br>(Sanz & Fersht) <sup>f</sup> |
|-----------------------|---|------------|---------------|---|---|---------------------------------------|--|
| T105V                 | 3.42                                      | 0.3        | 3.50          | 0.062   | N.D. <sup>g</sup>                                       | 0.14 ± 0.03                           | N.D.                                     |
|                       |   |            | 4.00          | 0.029   | N.D.  | 0.10 ± 0.02                           | N.D.                                     |
|                       |   |            | 4.50          | < 0.020   | N.D.  | N.D.                                  | N.D.                                     |
| 176T                  | 3.26                                      | 0.4        | 3.00          | 0.045   | 0.076   | 0.04 ± 0.01                           | 0.08 ± 0.02                              |
|                       |   |            | 3.50          | 0.042   | 0.034   | 0.06 ± 0.01                           | 0.05 ± 0.01                              |
|                       |   |            | 4.00          | < 0.020   | 0.014   | N.D.                                  | N.D.                                     |
| 14A/Y78F              | 3.21                                      | 0.0        | 3.25          | 0.196   | 0.218   | 0.39 ± 0.12                           | 0.46 ± 0.14                              |
|                       |   |            | 3.50          | 0.163   | 0.143   | 0.39 ± 0.13                           | 0.34 ± 0.11                              |
|                       |   |            | 3.75          | 0.106   | 0.093   | 0.29 ± 0.11                           | 0.25 ± 0.08                              |
|                       |   |            | 4.00          | 0.067   | 0.059   | 0.21 ± 0.08                           | 0.18 ± 0.06                              |
|                       |   |            | 4.25          | 0.037   | 0.037   | 0.13 ± 0.05                           | 0.13 ± 0.05                              |
| S92A                  | 3.14                                      | 0.4        | 3.00          | 0.035   | N.D.  | 0.03 ± 0.01                           | N.D.                                     |
| 14A/I51V              | 2.96                                      | 0.0        | 3.00          | 0.246   | 0.230   | 0.54 ± 0.16                           | 0.49 ± 0.17                              |
|                       |   |            | 3.25          | 0.164   | 0.155   | 0.41 ± 0.13                           | 0.38 ± 0.14                              |
|                       |   |            | 3.50          | 0.092   | 0.103   | 0.26 ± 0.09                           | 0.30 ± 0.11                              |
|                       |   |            | 3.75          | 0.064   | 0.068   | 0.22 ± 0.08                           | 0.23 ± 0.09                              |
|                       |   |            | 4.00          | 0.040   | 0.047   | 0.16 ± 0.06                           | 0.18 ± 0.07                              |
| D93N                  | 2.40                                      | 0.4        | 2.40          | < 0.020   | N.D.  | N.D.                                  | N.D.                                     |
| L14A                  | 2.29                                      | 0.5        | 2.50          | 0.035   | N.D.  | 0.02 ± 0.01                           | N.D.                                     |
|                       |   |            | 3.00          | < 0.020   | N.D.  | N.D.                                  | N.D.                                     |
| 14A/I25V<br>I51V/Y78F | 1.84                                      | 0.1        | 2.00          | 1.197   | 1.165   | 0.74 ± 0.10                           | 0.71 ± 0.14                              |
|                       |   |            | 2.25          | 0.911   | 0.852   | 0.62 ± 0.09                           | 0.56 ± 0.12                              |
|                       |   |            | 2.50          | 0.716   | 0.616   | 0.56 ± 0.09                           | 0.45 ± 0.10                              |
|                       |   |            | 2.75          | 0.475   | 0.440   | 0.39 ± 0.07                           | 0.35 ± 0.08                              |
|                       |   |            | 3.00          | 0.387   | 0.310   | 0.38 ± 0.07                           | 0.28 ± 0.06                              |

<sup>a</sup>Midpoint of urea equilibrium denaturation. <sup>b</sup> $\phi_1$  is defined as  $\phi_1 = 1 - \Delta\Delta G_{I-F} / \Delta\Delta G_{U-F}$ , where  $\Delta\Delta G = \Delta G$  (wild-type) -  $\Delta G$  (mutant) [1]. <sup>c</sup>Experimental error in  $k_{obs}$  is equal or less than 1%. <sup>d</sup>Calculated using eq. (5) and  $K_{IU}$  data from [3]. <sup>e</sup>Calculated using eq. (4).  $k_u$  data were taken from [3]. <sup>f</sup>Calculated using  $\Delta G_{U-F}$  and  $\Delta G_{I-F}$  from [3]. <sup>g</sup>N.D. Not determined.

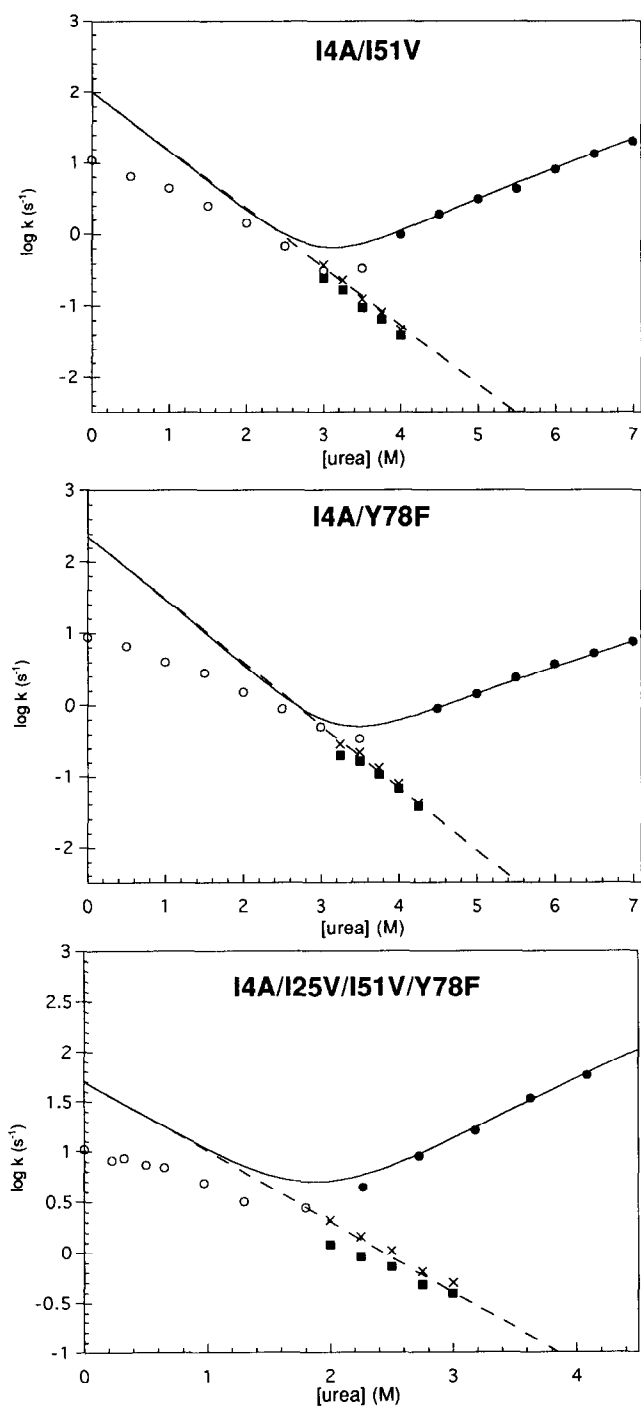


Fig. 2. Kinetic data of barnase mutants. Measured rate constants are plotted against the concentration of urea. (○), refolding of barnase; (●), unfolding of barnase; (■), rate constants measured upon binding of barstar ( $\log k_{\text{obs}}$ ); (x),  $\log k_F$  as calculated with eq. (5) using the  $K_{\text{IU}}$  values from Sanz and Fersht [3]. Solid line: expected kinetic behaviour for a two-state equilibrium ( $\log (k_F + k_u)$ ); dashed line, extrapolation of  $\log k_F$  to high [urea].

allows the direct calculation of  $K_{\text{IU}}$  constants, and by checking the  $k_F$  branch of the two-state model it also confirms the calculated value of  $k_u$  in water.

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