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Metal binding kinetics of bi-Histidine sites used in ψ -analysis: Evidence for high energy protein folding intermediates

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Abstract

The zinc-specific fluorophore, Zinpyr-1, is used in competition assays to determine the kinetic and thermodynamic parameters of Zn^{2+} binding to engineered bi-Histidine sites located in Ubiquitin and the B domain of protein A (BdpA). These binding sites are used in ψ -analysis studies to investigate structure formation in the folding transition state identified by the change in folding rate upon the addition of metal ions. For Ubiquitin, the on-rate binding constant and binding affinity for a site located along an α -helix are measured to be $\sim 10^7 \text{ M}^{-1}\text{s}^{-1}$ and $3 \mu\text{M}$, respectively. For a site located across two β -strands, the metal binding affinity was too weak to measure in the dye competition assays ($K_d > 55 \mu\text{M}$). The equilibrium-determined values for the Zn^{2+} -induced stabilization of Ubiquitin and BdpA match the values derived from changes in the global folding and unfolding rates. Therefore, metal-ion binding is in fast equilibrium during the transit over the free energy barrier. Accordingly, the folding rate must be slower than the product of the fractional population of a high energy intermediate with the metal site formed and the metal binding on-rate constant. The known folding rate of 20 s^{-1} at 1.5 M guanidinium chloride in $400 \mu\text{M} \text{ Zn}^{2+}$ provides an upper bound for the stability of such intermediates, $\Delta G_{\text{U-I}} < +4 \text{ kcal}\cdot\text{mol}^{-1}$. These results support a view of the apparent two-state protein folding reaction surface as a fast pre-equilibrium between the denatured state and a series of high energy species. The net folding rate is a product of the equilibrium constant of the highest energy species and a transmission rate. For Ubiquitin, we estimate the transmission rate to be $\sim 10^4 \text{ s}^{-1}$. Implications to the role of unfolded chain diffusion on folding rates and barrier heights are discussed.

Keywords

Psi-analysis; Ubiquitin; free-energy barrier; kinetics

Metal binding is ubiquitous in biology, being important for folding, stability, transport and catalysis. Systems utilizing metal ions include metalloregulatory proteins, hemoglobin, tertiary RNAs, transcription factors and DNA repair machinery. One important example is human prion protein, PrP, which binds metals via Histidine residues in an octapeptide repeat (I). The conformational change from α -helix to β -sheet associated with amyloid formation in PrP abolishes Zn^{2+} and Mn^{2+} binding while increasing the affinity for nickel and potentially resulting in a loss of metalloregulatory function and the neurotoxicity of prions. The delineation of the kinetic and thermodynamic properties of metal binding proteins is essential to identifying how deficiencies in metal binding can disrupt cellular function and lead to disease.

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Many metal binding studies have been conducted using zinc finger proteins, which fold into a DNA-binding competent structure upon binding Zn^{2+} . These studies reveal that many metal-binding proteins bind with an on-rate constant between 10^6 (2, 3) and $10^9 \text{ M}^{-1}\text{s}^{-1}$ (4-6). These rate constants are often measured by competition assays in which pre-bound cobalt is chased with Zn^{2+} and the dissociation of Co^{2+} is monitored via changes in absorbance at 640 nm (3).

In the current study, we determine the rate and equilibrium constants of metal binding to the folded and denatured states of two metal-binding bi-Histidine (biHis) variants of the 76-residue α/β protein, Ubiquitin (Ub). Our study is motivated by the application of ψ -analysis (7-13), a method to characterize the folding TS analogous to mutational ϕ -analysis (14-16). In ψ -analysis, biHis metal ion binding sites are individually introduced at known positions throughout the protein to stabilize secondary and tertiary structures. The addition of divalent metal ions stabilizes the interaction between the two known Histidine positions. The stabilization is controlled as a continuous function of metal concentration. The degree to which the transition state ensemble (TSE) is stabilized by metal ions ($\Delta\Delta G_f^\ddagger$) relative to the

change in native state stability ($\Delta\Delta G_{eq}$) is quantified with $\psi = \frac{\partial\Delta\Delta G_f^\ddagger}{\partial\Delta\Delta G_{eq}}$, the instantaneous slope of the Leffler plot of $\Delta\Delta G_f^\ddagger$ versus $\Delta\Delta G_{eq}$ (17). The slope in the limit of no perturbation (i.e. no metal) is termed the ψ_0 -value. A ψ_0 of zero or one indicates that the biHis site is absent or formed in a native-like manner in the TSE, respectively.

After measuring ψ -values at fourteen sites across the protein Ubiquitin (Ub), we concluded that Ub's TSE is highly structured with a native-like topology. In particular, five biHis sites had ψ_0 of unity, indicating native-like binding affinities in the TSE. These sites define a minimal obligate core consisting of the carboxy-terminus of Ub's α -helix and four aligned β -strands. Around this core, another six sites had intermediate ψ_0 , indicative of sites which are fractionally populated or distorted in the TSE (9, 11, 18).

Of particular interest here is the mode of metal-induced stabilization of the native state and the TSE. This stabilization reflects the differential ion binding affinity of a biHis site in the denatured state and either the native or the TSE:

$$\Delta\Delta G_{eq}([Me^{2+}]) = -RT \ln \left(1 + \frac{[Me^{2+}]}{K_{eq}^{S,N}} \right) / \left(1 + \frac{[Me^{2+}]}{K_{eq}^{S,U}} \right) \quad \text{Eq. 1a}$$

$$\Delta\Delta G_f^\ddagger([Me^{2+}]) = -RT \ln \left(1 + \frac{[Me^{2+}]}{K_{eq}^{S,TS}} \right) / \left(1 + \frac{[Me^{2+}]}{K_{eq}^{S,U}} \right) \quad \text{Eq. 1b}$$

where $K_{eq}^{S,N}$, $K_{eq}^{S,U}$, and $K_{eq}^{S,TS}$ are the metal ion binding affinities for the biHis site in the native, unfolded, and TS. For a native-like site in the TS, $K_{eq}^{S,TS} = K_{eq}^{S,N}$, $\Delta\Delta G_f^\ddagger = \Delta\Delta G_{eq}$, and hence, $\psi_0 = 1$.

Rather than measure the metal ion binding affinities directly, the change in equilibrium stability is determined from the change in folding and unfolding rates of the biHis mutants in the presence of increasing ion concentrations (see Fig. 3B in Ref. (10)),

$$\Delta\Delta G_{eq}([Me^{2+}]) = RT \ln \left(\frac{k_f^{Me^{2+}}}{k_f^0} \frac{k_u^0}{k_u^{Me^{2+}}} \right) \quad \text{Eq. 2}$$

where k_f^0 and k_u^0 are the folding and unfolding rates in the absence of metal, respectively. Equation 2 implicitly assumes that metal ion binding is in fast equilibrium relative to the over-all folding rates. This assumption is supported by the agreement between the equilibrium and kinetically determined values of $\Delta\Delta G_{eq}([Co^{2+}])$ for a biHis site in Ub (a difference less than 10%, Fig. 2C in Ref. (10)). That is, ion binding exerts its full thermodynamic effect prior to or during the passage over the kinetic barrier (Fig. 1). If equilibration occurs during the passage, then the site must be present often enough that the metal ions are able to bind prior to over-all folding, i.e.

$$k_f < K_{eq}^{U-I} \cdot k_{on}^{biHis} \cdot [Zn^{2+}] \quad \text{Eq. 3}$$

where k_f is the folding rate, K_{eq}^{U-I} is the equilibrium constant for the lowest energy state where the biHis site is binding competent, and k_{on}^{biHis} is the on-rate of metal binding.

Here we test whether the affinities of the biHis sites determined from the change in stability correspond to the actual binding affinities. We examine biHis binding sites (Fig. 1B), site *h* spanning β -sheets, $\beta 3$ - $\beta 4$ (residues 42,70), and site *k* on the N-terminus of the α -helix (residues 24,28) with ψ_o -values of 0.3 and 0.2, respectively (10). The fractional ψ_o indicate that these sites may be formed near the apex of the free energy reaction surface. We also include results for two helical sites having fractional ψ_o on the three-helix bundle BdpA (residues 11,15; $\psi_o = 0.2$; residues 29,33; $\psi_o = 0.5$) (13).

The major challenge in our study is that the metal binding of the biHis sites cannot be studied directly. Zn^{2+} is spectroscopically silent as are the standard spectroscopic probes, Co^{2+} and Ni^{2+} with biHis sites (3, 19). The bivalent nature of the surface sites impedes their ability to “lock” Co^{2+} or Ni^{2+} into a consensus binding geometry unlike most naturally occurring multivalent sites. The lack of absorbance signal for Zn^{2+} necessitates an indirect approach to measuring metal binding.

Accordingly, we employ a series of kinetic and equilibrium competition measurements to determine metal binding affinities and rates. We use the Zn^{2+} -specific fluorophore, Zinpyr-1 (ZP-1, Fig. 2) in competition experiments to obtain the metal on-rates and affinities to the biHis sites. We measure the on-rate of Zn^{2+} to the α -helical site in Ub to be $k_{on} \sim 3 \times 10^7$

$M^{-1}s^{-1}$ with binding affinity $K_{eq}^{S,N} \sim 2 \mu M$. Furthermore, the helical site retains some affinity in the unfolded state ($K_{eq}^{S,U} \sim 100 \mu M$). These binding affinities are quantitatively consistent with the metal-induced stabilization of the protein, determined by changes in either folding rates or equilibrium denaturation profiles. This correspondence indicates that metal binding establishes an equilibrium faster than the over-all folding rate. While the Zn^{2+} affinity to the β -sheet site is too weak to measure in the dye competition assays, the metal-induced stabilization for this site was the same whether measured kinetically (changes in folding rates) or thermodynamically (changes in denaturation midpoint). As discussed above, the associated biHis site must be formed often enough for metal binding to equilibrate. For a biHis site formed in an intermediate near the top of the free energy barrier, this information can be used to assign an upper bound on the stability of the intermediate relative to the unfolded state. Using our measured metal on-rate and Eq. 3, we estimate the upper bound of the stability of such an intermediate to be $\Delta G_{U-I} < +4 \text{ kcal} \cdot \text{mol}^{-1}$.

Results

Effective Binding Constants from Metal-Dependent Folding Studies

Previous studies on Ub (10) found that the change in protein stability as a function of divalent metal ion was well described by the difference between the binding-induced stabilization of the native state and the stabilization of the denatured state, as given by Eq. 1a. We first tested whether this behavior is maintained in the case of Zn^{2+} for two biHis sites on Ub, site *h* across two β -strands (R42H/V70H) and site *k* on the amino-terminus of the α -helix (E24H/A28H). The equilibrium stability as a function of $[\text{Zn}^{2+}]$ is obtained from the changes in the folding and unfolding rates according to Eq. 2. Values of k_f and k_u are obtained from kinetic measurements under strongly folding and unfolding conditions, either 2–4 M or 6 M guanidinium chloride (GdmCl), respectively (data not shown).

The resulting values of $\Delta\Delta G_{eq}([\text{Zn}^{2+}])$ are fit to two different models to determine whether ion binding to the denatured state is a significant factor for the two sites (Table 1, Fig. 3). The incorporation of denatured state binding marginally improves the fit to the β -sheet site *h* data. The native binding affinity, $K_{eq}^{S,N}$, is $32 \pm 3 \mu\text{M}$ while the denatured state binds weakly, if at all, $K_{eq}^{S,U} = 294 \pm 73 \mu\text{M}$. For the α -helical site *k*, however, the inclusion of denatured state binding significantly improves the fit, yielding $K_{eq}^{S,N} = 3.2 \pm 0.2 \mu\text{M}$ and $K_{eq}^{S,U} = 85 \pm 13 \mu\text{M}$. To test the generality of the results, the measurements are repeated on BdpA with a biHis site on the amino terminal helix (Q11H/Y15H). Values of k_f and k_u are obtained from kinetic measurements under strongly folding and unfolding conditions, i.e. 2.4 M and 5.5 M GdmCl, respectively (data not shown). As with the helical site on Ub, the fit of $\Delta\Delta G_{eq}([\text{Zn}^{2+}])$ improves substantially with a model that includes denatured state binding with affinities $K_{eq}^{S,N} = 16 \pm 1 \mu\text{M}$ and $K_{eq}^{S,U} = 65 \pm 6 \mu\text{M}$.

Metal Binding is in Fast Equilibrium

To investigate whether the binding of metal ions to biHis sites is in fast equilibrium relative to over-all folding rates, we compared the values of the equilibrium stability determined from the aforementioned changes in folding rates to the values obtained by equilibrium chemical denaturation measurements. The GdmCl denaturation profiles are obtained for multiple Zn^{2+} concentrations (Fig. 4, inset). For both Ub sites, the equilibrium and kinetically determined values of $\Delta\Delta G_{eq}([\text{Zn}^{2+}])$ are in very good agreement with a deviation of less than $0.2 \text{ kcal}\cdot\text{mol}^{-1}$. For the eight biHis sites located on the three helices of BdpA, the stability increased between 0.3 and $1.7 \text{ kcal}\cdot\text{mol}^{-1}$ in the presence of $1 \text{ mM } \text{Zn}^{2+}$ (13). Nevertheless, for any given site, the equilibrium and kinetically determined values are very similar (deviation less than $0.2 \text{ kcal}\cdot\text{mol}^{-1}$). Hence, prior to or during the passage over the kinetic barrier, metal ion binding is able to exert its full thermodynamic effect on the weakly populated intermediates containing a binding competent biHis site.

Determination of Metal Binding Equilibrium and Rate Constants

To directly measure the metal binding affinity to a biHis site, the Zinc-specific fluorophore ZP-1 is used in competition assays. This fluorophore is based on the quinoline family of metal-specific fluorophores (Fig. 2a). Metal-binding fluorophores such as Zinpyr-1 were originally developed for measuring the intracellular concentration and localization of metals in vivo via fluorescence microscopy. In this study, however, we utilize the high specificity and affinity of ZP-1 for Zn^{2+} to act as a sink for free Zn^{2+} in kinetic and equilibrium competition assays.

Upon chelation of a single Zinc ion, ZP-1's excitation and emission maxima are 505 and 520 nm, respectively (Fig. 2b). The bimolecular on-rate constant of Zn^{2+} binding ZP-1, k_{on}^{ZP} , is obtained by measuring the rate of ZP-1: Zn^{2+} complex formation over a range of (excess) Zn^{2+} concentrations. Each of the reaction curves are fit to a single exponential to yield the apparent rate constant, k_{obs} (data not shown). The slope of a linear fit of k_{obs} ($[\text{Zn}^{2+}]$) yields the bimolecular on-rate constant of $k_{on}^{ZP} = 3.7 \pm 0.1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ in 0 M GdmCl.

The binding of Zn^{2+} to ZP-1 depends on buffer conditions. Specifically, the on-rate in the presence of 1.5 and 6 M GdmCl is $k_{on}^{ZP} = 6.8 \pm 0.2 \times 10^6$ and $1.1 \pm 0.2 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$, respectively (data not shown). This non-monotonic behavior is due to the ionic strength of the buffer, rather than a denaturant effect. Measurements are repeated in the presence of NaCl rather than GdmCl. The on-rate constant increases 2-fold until ~2.5 M and then gradually decreases at higher $[\text{NaCl}]$ (data not shown).

We designed two competition assays using ZP-1 to measure the metal binding affinity to a biHis site on the proteins. The first is an equilibrium competition (EqComp) assay where 0-300 μM Ub is pre-equilibrated with 1 μM Zn^{2+} before mixing with 3 μM ZP-1. The second is a kinetic competition (KinComp) assay where all three components are simultaneously mixed. In the EqComp assays, the fraction of Zn^{2+} not bound to the biHis site prior to the addition of dye, $[\text{Zn}^{2+}]_{free}$, determines the initial ZP-1: Zn^{2+} binding rate, k_{obs} . From the decrease in k_{obs} , relative to the rate in the absence of protein, the equilibrium binding constant for the biHis site is determined. In the KinComp assay, however, the observed ZP-1: Zn^{2+} binding rate is influenced by the rate of metal binding to Ub in addition to the binding affinity. The ZP-1: Zn^{2+} on-rate is dependent on the total amount of Zn^{2+} , $[\text{Zn}^{2+}]_{total}$, as well as the Ub: Zn^{2+} on- and off-rates.

The concentrations in the EqComp assay are such that $[\text{ZP-1}] = 3[\text{Zn}^{2+}]$, a condition where the dye-metal binding rate is under near pseudo-first order conditions (20). The dye-metal off-rate is slow ($k_{off} < 0.01 \text{ s}^{-1}$) and so formation of ZP-1: Zn^{2+} is effectively irreversible over the course of the measurement. The dye-metal on-rate, k_{obs} , may be approximated by $k_{on}^{ZP}[\text{Zn}^{2+}]_{free}$ such that

$$k_{obs} \sim k_{on}^{ZP} \frac{-B + \sqrt{B^2 - 4C}}{2} \quad \text{Eq. 4}$$

where $B = K_{eq}^S + [\text{Ub}]_{total} - [\text{Zn}^{2+}]_{total}$, $C = -K_{eq}^S[\text{Zn}^{2+}]_{total}$, and K_{eq}^S is the binding affinity to the biHis site.

The binding of Zn^{2+} to the Ub β -sheet site is considerably weaker ($> 55 \mu\text{M}$), and matches control experiments performed with a Ub lacking a biHis site. So only the results for the α -helical sites are reported under native-like conditions. For either Ub or BdpA biHis mutants, increasing protein concentrations (up to 300 μM) reduce the observed ZP-1: Zn^{2+} binding rate by nearly ten-fold. The fitting of the observed rates to Eq. 4 for the helical sites on Ub (in 1.5 M GdmCl) and BdpA (N29H/Q33H, 2.2 M GdmCl) yields binding affinities of $K_{eq}^{S,N} = 1.8 \pm 0.2$ and $3.2 \pm 0.4 \mu\text{M}$, respectively (Fig. 5, Table 1).

In the KinComp experiments, the protein and Zn^{2+} are not pre-equilibrated, but are simultaneously mixed with ZP-1. As a result, the observed ZP-1: Zn^{2+} binding rate depends on both the Zn^{2+} on-rate and its affinity for the biHis site on the protein. The dye binding trace is monitored with increasing concentrations of protein (Fig. 6). These experiments are performed in the presence of 1.5 M GdmCl to mimic the conditions of previous metal-

dependent folding studies (10). The observed rate of ZP-1:Zn²⁺ formation decreases with increasing concentration of Ub as the protein reduces the free Zn²⁺ concentration.

To extract kinetic and thermodynamic information from the KinComp experiments, the kinetic modeling software, DynaFit (21), is used to globally fit these data to the following model: The value of k_{on}^{ZP} is fixed to the value determined from the independent measurements without protein, while the Ub on- and off-rate constants are allowed to vary during the fit. The kinetic parameters for the α -helical site k are

$k_{on}^{biHis} = 2.7 \pm 2.2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ and $k_{off}^{biHis} = 76 \pm 11 \text{ s}^{-1}$ (Table 2). The resulting affinity, $K_{eq}^{S,N} = 2.8 \pm 2.3 \text{ } \mu\text{M}$, is consistent with the value determined in the other measurements (Table 1).

Investigation of Denatured State Binding

As denatured state metal binding could influence the interpretation of ψ -analysis, we directly tested for such binding. As previously discussed, the allowance for both folded and denatured state binding better describes the observed $\Delta\Delta G_{eq}([\text{Zn}^{2+}])$ data for the helical site (Eq. 1a, Fig. 3). This improvement suggests that biHis sites can bind metal in the denatured state for α -helical sites. To verify this observation, the rate and equilibrium constants of metal binding to Ub sites h and k are measured in 6 M GdmCl using KinComp assays (Fig. 6, Table 2) and Scheme 1. Under denaturing conditions, the concentration of β -sheet site h does not significantly affect the rate of ZP-1:Zn²⁺ complex formation ($K_{eq}^{S,U} > 240 \text{ } \mu\text{M}$). The rate of ZP-1:Zn²⁺ complex formation decreases in the presence of the α -helical site k concentration in both the folded and denatured states, specifically, $K_{eq}^{S,N} = 15.0 \pm 0.3 \text{ } \mu\text{M}$ and $K_{eq}^{S,U} = 118.00 \pm 0.02 \text{ } \mu\text{M}$. This retardation in rate indicates that the α -helical site is binding competent in the denatured state and competes with ZP-1.

These values for the binding affinities obtained from the KinComp assays are compared to the values obtained from metal-dependent folding studies and EqComp assays (Table 1). There is significant agreement between all three measurements. As observed in the ZP-1:Zn²⁺ binding experiments, the value of the on-rate constant of Zn²⁺ binding to Ub is slower at 0 M GdmCl than at 1.5 M GdmCl (5×10^6 versus $1.7 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$), which is consistent with the evidence that metal binding shows a non-monotonic dependence on ionic strengths with affinity peaking at ~2.5 M.

Discussion

In this study, we have employed kinetic and equilibrium competition assays using the Zinc-specific fluorophore, Zinpyr-1, to determine the thermodynamic and kinetic constants of Zn²⁺ binding to biHis sites on the surface of Ub and BdpA. Overall, we have seen that the binding affinities measured for the folded state and the denatured state recapitulate the change in global protein stability with metal, $\Delta\Delta G_{eq}([\text{Zn}^{2+}])$. This agreement supports our interpretation that the mechanism of metal-induced stability imparted by biHis sites to a protein reflects the difference of the binding affinities in the native and denatured states according to Eq. 1a.

Our results provide direct evidence that metal can bind to α -helical biHis sites in the denatured state. From the kinetic competition measurements, the α -helical site on Ub was found to have a metal binding affinity of 118 μM in the presence of 6 M GdmCl. In addition, denatured state binding was implicated by the improved fit using the linked equilibrium

expression (Eq. 1a) to the change in stability upon addition of metal for the helical sites on both Ub and BdpA (Fig. 2). Metal binding for the β -sheet site was weak in all conditions.

The difference between binding affinity in the denatured state for the helical and sheet biHis sites reflects their difference in sequence proximity. The helical site is sequence local ($i, i+4$) while the sheet site is non-local ($i, i+28$). The Histidines on the two strands are much less likely to be close enough to form a binding competent geometry in an otherwise denatured chain. There is no evidence that the binding for either of these “denatured” structures are native-like, though it seems likely that the two Histidines adopt a helical geometry as observed in isolated helices (22). However, the possibility that a short β -turn is transiently formed cannot be completely ruled out. For the helical sites, the binding constant in high denaturant likely represents the metal-induced stabilization of the site scaled by the equilibrium constant for the formation of the site (e.g. a turn of a helix, σ in Zimm-Bragg helix-coil theory (23)).

The observed on-rate constant of Zn^{2+} to a pre-organized biHis site is $k_{on}^{Ub} \sim 10^7 \text{ M}^{-1} \text{ s}^{-1}$. This value is very similar to that found by Bombarda et al., $3.9 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$, for bivalent Cys-His sites on HIV NCp7 (24), and by Hunt et al., $1 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ for biHis sites on wild type carbonic anhydrase (25). Studies of other bi-dentate sites indicate that these sites also bind with μM affinity (2, 3). The affinities are weaker than the nM-pM range found in other Zn-binding studies for multi-dentate binding sites (4-6).

Metal ion Binding is in fast equilibrium

For the two and nine measured biHis sites on Ub and BdpA, respectively, the change in protein stability due to Zn^{2+} binding determined by equilibrium denaturation profiles matches those values determined by the changes in the folding kinetics (Eq. 2). Therefore, ion binding is able to exert its full thermodynamic effect prior to or during the passage over the kinetic barrier. Binding competent states must populate often enough for the metal ions to establish an equilibrium, as given by the inequality in Eq 3.

The option of binding metal ions in the denatured state for the helical sites enables this inequality to be readily satisfied. Regardless of whether denatured state binding occurs in the case of high ψ -values, the formation of such sites must be relevant for the TSE as they have native or near-native binding affinity by this point on the reaction surface and reduce $\Delta G_f^\ddagger$, the height of the kinetic barrier. Generally, binding in the denatured state alone is insufficient to generate significant ψ values, and does not change their interpretation. A site which produces a kinetic response stabilizes on-pathway folding intermediates and the TSE.

For the long-range β -sheet biHis site, denatured state binding is very weak. Hence, it is unlikely that the establishment of the ion binding equilibrium prior to folding can be accounted for by denatured state binding. For long-range sheet sites with non-zero ψ , the sites form on the way up the kinetic barrier, yet are populated often enough that metal ions can bind and establish a thermodynamic equilibrium.

Folding landscapes and barrier heights

Despite the fact that protein folding is a physically complex process, many experiments over the last few decades have revealed that it is not kinetically complex. For many small proteins, the kinetics are well described with a single exponential, indicative of the free-energy surface being dominated by a single barrier (26). Crossing this barrier likely requires a series of coordinated events in a largely sequential manner (Fig. 7). But, directly examining the transiently populated species on the pre-TS side of a major folding barrier is difficult. The use of biHis sites provides us with an opportunity to explore such events. As

the chain traverses the folding landscape, the biHis sites with non-zero ψ are present often enough to establish a measurable binding affinity. For biHis sites where denatured state binding is inconsequential, folding can be described as series of thermodynamic wells where these biHis sites are formed in fast equilibrium with each other and the denatured state. Further, the associated intermediates establish a rapid pre-equilibrium faster than the overall folding reaction.

We estimate the free energy associated with such a high energy intermediate formed in rapid pre-equilibrium. The β -sheet site h investigated here is an example of such an intermediate. It has a fractional ψ -value, binds metal weakly if at all in the denatured state, and likely forms only near the top of the barrier. This site connects strands 3 and 4, the last two elements to associate on the up-hill side of the barrier. Since we were unable to directly measure the on-rate for site h , we use the measured rate for the helical site as an estimate. Presumably, this estimate is an upper bound since the β -sheet site has a weaker affinity for metal. Using $k_{on}^{biHis} \sim 3 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ and $k_f = 20 \text{ s}^{-1}$ in 1.5 M GdmCl, we estimate that in 400 μM Zn^{2+} , the intermediate containing this biHis site is formed with an equilibrium constant of $K_{eq}^{U-I} > 0.002$, or $\Delta G_{U-I} < +4 \text{ kcal}\cdot\text{mol}^{-1}$. Hence, the 4 $\text{kcal}\cdot\text{mol}^{-1}$ value provides an estimate of the free energy of a well near the top of the reaction surface.

We stress that the energy of the species is not the absolute barrier height, $\Delta G_f^\ddagger$, as the overall barrier height is the energy of the intermediate plus the final kinetic barrier height. Equivalently, the folding rate is given by the rate of crossing the final rate-limiting barrier scaled by the fraction of time the intermediate is formed, i.e. $k_f = K_{eq}^{U-I} k_{trans}$. We use the above values for k_f and K_{eq}^{U-I} to estimate that $k_{trans} \sim 1 \times 10^4 \text{ s}^{-1}$. Although the actual event associated with this final step is unknown, information about the Ub pathway garnered from ψ -analysis (10) suggests a folding event involving either the consolidation of the existing four strand/helix TS structure defined by the sites with non-zero ψ , or the initial formation of the 3_{10} helix (the next major structural element to form after the TS). Our k_{trans} rate is slower than the rate measure by Gai *et al.* of helix formation for naturally-occurring sequences (27), which is, in turn, an order of magnitude slower than for alanine-rich helices. Our lower transmission rate may be due to the complicated nature of the remaining folding steps (e.g. the 3_{10} helix folds from an interior, closed loop).

The view of folding as a series of rapid pre-equilibrium folding events is consistent with clustering analysis of transient species by Shakhovich *et al.* (28) and Pande *et al.* (29) while contrasting with the simpler model of a reaction surface containing only the unfolded and native state wells separated by a single barrier. While the view adopted here may seem to be only a refinement of the simple model, the differences have implications to the estimation of absolute barrier heights from folding rates. Various groups have proposed strategies for elucidating the relationship between the free-energy surface, the folding rate, and the height of the major barrier, e.g.,

$$k_f = \frac{\omega_{\max}}{2\pi\omega_{\min}\tau_o} e^{-\Delta G^\ddagger/RT} \quad \text{Eq. 5}$$

where $\omega_{\min}$ and $\omega_{\max}$ are frequencies that characterize the curvature and ruggedness of the free energy surface in the harmonic well of the unfolded state and at the top of the barrier (30). These efforts are based on extensions of classical transition state theory (TST) (31, 32). In classical TST, the height of the barrier is estimated using the Arrhenius equation $k = A_0 e^{-\Delta G^\ddagger/RT}$ where the pre-exponential factor A_0 is the attempt frequency, which, in the case of protein folding, is the peptide reconfiguration rate (33). In the case of rapid pre-

equilibrium between the unfolded state and partially folded, high energy intermediates, the reaction surface's curvature and ruggedness (i.e. diffusion constant) near the unfolded state does not factor into the over-all folding rate. Rather, $\omega_{\min}$ will depend on the properties of the energy well populated just prior to passage across the major barrier.

Schuler *et al.* (33) estimated the free energy barrier using single molecule FRET in conjunction with Kramers' theory assuming a single barrier. Based on the rate of collapse in unfolded *T. maritima* cold shock protein, they estimated the free energy barrier height to be 2-7 kcal·mol⁻¹, with an attempt frequency between (0.3 μs)⁻¹ and (0.2 ms)⁻¹. Studies of the temperature dependence of the folding kinetics and ϕ -values of hYAP WW domain by Gruebele *et al.*, calculated the barrier to be approximately 2.6 kcal·mol⁻¹ (34). A direct comparison of these barrier heights with the energy of the species containing biHis site *h* is inappropriate as the intermediate represents a thermodynamic well rather than a barrier height. Nevertheless, we combine the energy of this intermediate with our estimates of $k_{\text{trans}} \approx (100 \mu\text{sec})^{-1}$ and $A_0 \approx (1 \mu\text{sec})^{-1}$ and calculate an upper bound to the barrier height for Ubiquitin, i.e. $\Delta G_f^\ddagger < 7 \text{ kcal} \cdot \text{mol}^{-1}$ (in 1.5 M GdmCl).

Conclusion

We have developed a method of determining the kinetic and thermodynamic constants of metal-binding to bivalent biHis sites on the surface of Ubiquitin and the B domain of protein A. The method has applicability for studying metal binding in systems that cannot be studied by conventional spectroscopic methods. Our experiments gained access to information previously unavailable for weak, bivalent binding sites. We use this information to determine the energetics of pre-transition state conformations of the Ubiquitin folding pathway. We find that in 1.5 M GdmCl, a high energy intermediate with the amino and carboxy terminal strands formed exists near the top of the Ub folding reaction surface. The stability of this intermediate is estimated to be $\Delta G_{\text{U-I}} < +4 \text{ kcal} \cdot \text{mol}^{-1}$ (relative to the denatured state).

The concordance between the direct binding measurements and the indirect methods based on changes in protein stability demonstrate that metal binding is well described by a linked equilibrium relating the difference in binding affinities in the folded and denatured states. More importantly, this binding is in fast equilibrium relative to the over-all folding rates. Hence, for apparent two-state folding, the simplified reaction surface with a single barrier is better depicted as one containing specific intermediates (i.e. thermodynamic wells) which rapidly equilibrate prior to the final folding step. Due to the rapid pre-equilibrium, states near the top of the major barrier rather than the denatured state determine the relevant chain diffusion constant (i.e. ruggedness of the landscape) in applications of Kramers theory.

Material and Methods

Protein purification

All Ubiquitin mutants were purified via expression in BL(21)DE3 cells. Ubiquitin mutants were prepared and purified as previously described (10). Verification of the correct Ubiquitin mutant is accomplished using mass spectroscopy. All relevant fractions were lyophilized and stored in sealed 50 mL conical tubes at room temperature. The BdpA mutants were prepared in the background of a pseudoWT (F14W/W15Y/N19H) taken from Sato *et al.* (35) (PDB 1SS1). The purification protocol of the protein mutants was similar to that reported above for Ubiquitin. However, the protocol differs in the FPLC step with the Sepharose Fast Flow SP column. The pH of the supernatant from the acid precipitation was not readjusted prior to running over the column, and a dH₂O + 0.01% TFA buffer was used

to pre-equilibrate the column. The elution of bound protein from the SP column was accomplished using a dH₂O/0.01% TFA/1M NaCl buffer.

Preparation of Zinc-Specific Fluorophore, Zinpyr-1, solutions

Zinpyr-1 (ZP-1) is a zinc-specific fluorescent dye with a dissociation constant, K_d , of 1 nM, excitation maximum at 505 nm, emission maximum of 530 nm and a quantum yield of $\phi = 0.87$ when zinc is bound (Neurobiotex, TX) (36, 37). ZP-1 was dissolved in DMSO by vortexing for 10-20 min., and the solution pulse spun on a table top centrifuge to pellet any undissolved particulate. This resulted in a stock concentration of > 1 mM, which was then diluted to working concentrations with 50 mM HEPES, 100 mM NaCl, pH 7.5. The concentration of ZP-1 was determined from UV-Vis absorbance at 515 nm using $\epsilon = 79,500$ M⁻¹cm⁻¹ (36, 37). A 1:2 ZP:Zn²⁺ binding stoichiometry was determined by titration of 1 μ M aliquots of Zn²⁺ to an 8 μ M ZP-1 solution. The decrease in absorbance at 515 nm was recorded and plotted against the concentration of metal. Similar studies using fluorescence showed a sigmoidal trend, which was not the result of cooperative binding, but an artifact of the increased transmittance of light at higher [Zn²⁺] and the resulting inner-filter effects. The second binding site of ZP-1 has a very weak ($K_d \sim 35$ μ M), and does not significantly bind Zn²⁺ at the low [Zn²⁺] conditions of the current studies (36).

Stopped-Flow Measurements

All kinetics data were collected using a rapid mixing Biologic SFM-4 stopped flow apparatus equipped with an Oriel mercury arc lamp and Hamamatsu photomultiplier tube with a customized, “teflon” light bevel to decrease reflected incident light. Fluorescence was excited at 283nm or 486nm for Ubiquitin folding or ZP-1 metal binding, respectively, and cut-off filters were used to collect fluorescence emission above 310 nm or 520 nm, respectively. Traces were fit to single or double exponential equations using BioKin fitting software.

Determination of the Rate Constants of Zn²⁺ binding to Zinpyr-1

To measure the intrinsic on-rate constant of Zn²⁺ to ZP-1, we performed stopped-flow experiments (as described above) to measure the kinetics of 0.147 μ M ZP-1 binding a range of Zn²⁺ concentrations from 0 to 1 mM in the presence of 0, 1.5 or 6 M GdmCl. The concentration of Zn²⁺ stocks was verified by ICP-MS (Stat Analysis Corp., Chicago, IL). The traces were fit to a single exponential equation and the values of k_{obs} plotted as a function of [Zn²⁺]. A linear fit of these data provided the value of the intrinsic bimolecular on-rate constants of ZP-1 binding Zn²⁺ at each concentration of denaturant, which were then used in the global fitting analysis described below. Control experiments with a Ub variant lacking a biHis site indicated that the dye interacts weakly with protein. This interaction increased ZP-1's fluorescence while decreasing the Zn²⁺ on-rate and binding affinity. It was necessary to wash the lines with buffer after every 4-6 shots to clear residual ZP-1 from the lines. In rare cases, a mixture of 50% Ethanol, 30% Methanol, and 10% DMSO, followed by excessive washing with buffer, was needed to fully cleanse the lines.

The on-rate constant and off-rate constant of ZP-1 also were determined by competition assay with EGTA based on experiments by Jackson, *et al.* (38). Using stopped-flow to measure kinetics, 20 μ M ZP-1 and 20 μ M ZnCl were pre-equilibrated and then challenged with 0-10 mM EGTA (data not shown). In the limit of high EGTA, the observed decrease in dye fluorescence is the off-rate of the metal. The kinetic data were fit to single exponential equations and the resulting k_{obs} plotted against the concentration of EGTA. The resulting hyperbola was fit according to previously published data (38).

Kinetic Competition (KinComp) Experiments

In order to measure the K_d and rate constants of metal binding to Ub, the three reaction components, ZP-1, Ub, and ZnCl_2 , were rapidly mixed such that the final concentrations are 3 μM , 0-200 μM , and 1 μM , respectively. In the stopped-flow protocol, the protein and ZP-1 were mixed prior to the addition of ZnCl_2 . The fluorescence signal was recorded for 0.5-5 seconds after final mixing. These conditions ensured that the concentration of Zn^{2+} is limiting and, hence, ZP-1 and Ub compete for the metal ion. These experiments were performed in 0, 1.5 M and 6 M GdmCl to investigate both folded and denatured state binding. The values of the unknown rate constants, k_{on}^{Ub} and k_{off}^{Ub} were determined by globally fitting kinetic traces using the program DynaFit (BioKin Ltd., WA)(21) to the differential equations derived from the reaction depicted in Scheme 1.

For experiments at different [GdmCl], the independently determined values for k_{on}^{ZP} (above) for the appropriate [GdmCl] ($4 \times 10^6 \text{M}^{-1}\text{s}^{-1}$ for 0 M GdmCl, $8 \times 10^6 \text{M}^{-1}\text{s}^{-1}$ for 1.5 M GdmCl and $2 \times 10^6 \text{M}^{-1}\text{s}^{-1}$ for 6 M GdmCl) were used as the initial values in the fitting process. Although ZP-1 does not bind Zn^{2+} irreversibly, the off-rate is significantly longer than the length of these experiments, and inclusion of k_{off}^{ZP} in the global fit returned a calculated off-rate of zero while not affecting the other values. As a result, it was subsequently excluded from further calculations. For global analysis of experiments performed at different GdmCl concentrations, the on-rate constant of Zn^{2+} binding ZP-1 was set to the value determined by linear titration of Zn^{2+} with ZP-1 in the presence of the appropriate GdmCl concentration. Multiple metal models were tested as well, but resulted in fits with large error and poor residuals, so only the single-Zinc model was used. Scripts for the global fit analysis can be found in Supplemental information.

Equilibrium Competition (EqComp) Experiments

Protein initially at 0-200 μM in 2 μM ZnCl_2 was diluted 1:2 upon rapid mixing with 3 μM ZP-1 (final concentration) via stopped-flow as described above and the fluorescence recorded for 0.5-5 seconds. The resulting traces were fit to single-exponential functions and the resulting k_{obs} was plotted versus [Ub]. The affinity was determined by fitting $k_{obs}([\text{Ub}])$ with the function given in Eq. 4.

Equilibrium measurements

CD measurements were conducted using a Jasco 715 spectropolarimeter with a 1 cm pathlength. The CD was measured at $\lambda=222$ or 226 nm with a 2-5 nm resolution for 2-10 μM protein. Ub and BdpA measurements were conducted in 50 mM HEPES, 100 mM NaCl, pH 7.5 and pH 7.7 respectively. Ub and BdpA measurements were carried out at 20°C and 10°C, respectively. Fluorescence excitation and emission spectra of ZP-1 were obtained with a Horiba Jobin Yvon FluoroMax 3.

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ABBREVIATIONS

biHis

biHistidine

EqComp	equilibrium competition assay
GdmCl	guanidinium chloride
$K_{eq}^{S,N}, K_{eq}^{S,U}, K_{eq}^{S,TS}$	metal binding affinity of a biHis site in the native, denatured and transition state ensemble
K_{eq}^{U-I}	equilibrium constant of an intermediate
k_{trans}	rate of transmission over the free-energy barrier
TSE	transition state ensemble
EqComp	equilibrium competition assay
KinComp	kinetic competition assay
ZP-1	the fluorophore Zinpyr-1
K_{on}^{ZP}	on-rate of Zn^{2+} binding to ZP-1
k_{on}^{biHis}	on-rate of Zn^{2+} binding to a Ub biHis site
k_{off}^{biHis}	off-rate of Zn^{2+} binding to a biHis site
Ub	mammalian Ubiquitin
BdpA	B domain of protein A
$\Delta\Delta G_{eq}$	change in free energy of the protein
$\Delta\Delta G_f^\ddagger$	change in free energy of the transition state of a folding pathway

References

1. Jackson GS, Murray I, Hosszu LL, Gibbs N, Waltho JP, Clarke AR, Collinge J. Location and properties of metal-binding sites on the human prion protein. *Proc Natl Acad Sci U S A*. 2001; 98:8531–8535. [PubMed: 11438695]
2. Gao H, Yu Y, Leary JA. Mechanism and kinetics of metalloenzyme phosphomannose isomerase: measurement of dissociation constants and effect of zinc binding using ESI-FTICR mass spectrometry. *Anal Chem*. 2005; 77:5596–5603. [PubMed: 16131071]
3. Shi Y, Beger RD, Berg JM. Metal binding properties of single amino acid deletion mutants of zinc finger peptides: studies using cobalt(II) as a spectroscopic probe. *Biophys J*. 1993; 64:749–753. [PubMed: 8471726]
4. Mely Y, Cornille F, Fournie-Zaluski MC, Darlix JL, Roques BP, Gerard D. Investigation of zinc-binding affinities of Moloney murine leukemia virus nucleocapsid protein and its related zinc finger and modified peptides. *Biopolymers*. 1991; 31:899–906. [PubMed: 1912345]
5. Mely Y, De Rocquigny H, Morellet N, Roques BP, Gerard D. Zinc binding to the HIV-1 nucleocapsid protein: a thermodynamic investigation by fluorescence spectroscopy. *Biochemistry*. 1996; 35:5175–5182. [PubMed: 8611501]
6. Bombarda E, Cherradi H, Morellet N, Roques BP, Mely Y. $Zn(2+)$ binding properties of single-point mutants of the C-terminal zinc finger of the HIV-1 nucleocapsid protein: evidence of a critical role of cysteine 49 in $Zn(2+)$ dissociation. *Biochemistry*. 2002; 41:4312–4320. [PubMed: 11914077]
7. Pandit AD, Krantz BA, Dothager RS, Sosnick TR. Characterizing protein folding transition states using Psi-analysis. *Methods Mol Biol*. 2007; 350:83–104. [PubMed: 16957319]
8. Sosnick TR, Krantz BA, Dothager RS, Baxa M. Characterizing the Protein Folding Transition State Using psi Analysis. *Chem. Rev*. 2006; 106:1862–1876. [PubMed: 16683758]

9. Sosnick TR, Dothager RS, Krantz BA. Differences in the folding transition state of ubiquitin indicated by phi and psi analyses. *Proc. Natl. Acad. Sci. U S A.* 2004; 101:17377–17382. [PubMed: 15576508]
10. Krantz BA, Dothager RS, Sosnick TR. Discerning the structure and energy of multiple transition states in protein folding using psi-analysis. *J. Mol. Biol.* 2004; 337:463–475. [PubMed: 15003460]
11. Krantz BA, Dothager RS, Sosnick TR. Erratum to Discerning the structure and energy of multiple transition states in protein folding using psi-analysis. *J. Mol. Biol.* 2004; 347:889–1109.
12. Krantz BA, Sosnick TR. Engineered metal binding sites map the heterogeneous folding landscape of a coiled coil. *Nature Struct. Biol.* 2001; 8:1042–1047. [PubMed: 11694889]
13. Baxa M, Freed KF, Sosnick TR. Quantifying the Structural Requirements of the Folding Transition State of Protein A and Other Systems. *J. Mol. Biol.* 2008; 381:1362–1381. [PubMed: 18625237]
14. Matthews CR. Effects of point mutations on the folding of globular proteins. *Methods Enzymol.* 1987; 154:498–511. [PubMed: 3431461]
15. Fersht AR, Matouschek A, Serrano L. The folding of an enzyme. I. Theory of protein engineering analysis of stability and pathway of protein folding. *J. Mol. Biol.* 1992; 224:771–782. [PubMed: 1569556]
16. Goldenberg, DP. Mutational Analysis of Protein Folding and Stability. In: Creighton, TE., editor. *Protein Folding.* W. H. Freeman; New York: 1992. p. 353-403.
17. Leffler JE. Parameters for the description of transition states. *Science.* 1953; 107:340–341. [PubMed: 17741025]
18. Baxa M, Freed KF, Sosnick TR. Psi-Constrained Simulations of Protein Folding Transition States: Implications for Calculating Phi. *J. Mol. Biol.* In press.
19. Frankel AD, Berg JM, Pabo CO. Metal-dependent folding of a single zinc finger from transcription factor IIIA. *Proc Natl Acad Sci U S A.* 1987; 84:4841–4845. [PubMed: 3474629]
20. Gutfreund, H. Cambridge University Press, University of Bristol; 1995. p. 60-62.
21. Kuzmic P. Program DYNAFIT for the analysis of enzyme kinetic data: application to HIV proteinase. *Anal Biochem.* 1996; 237:260–273. [PubMed: 8660575]
22. Ghadiri M, Choi C. Secondary structure nucleation in peptides - Transition-metal ion stabilized alpha-helices. *J. Amer. Chem. Soc.* 1990; 112:1630–1632.
23. Zimm GH, Bragg JK. Theory of the phase transition between helix and random coil in polypeptide chains. *J. Chem. Phys.* 1959; 31:526–535.
24. Bombarda E, Roques BP, Mely Y, Grell E. Mechanism of zinc coordination by point-mutated structures of the distal CCHC binding motif of the HIV-1 NCp7 protein. *Biochemistry.* 2005; 44:7315–7325. [PubMed: 15882070]
25. Hunt JA, Fierke CA. Selection of carbonic anhydrase variants displayed on phage. Aromatic residues in zinc binding site enhance metal affinity and equilibration kinetics. *J Biol Chem.* 1997; 272:20364–20372. [PubMed: 9252341]
26. Krantz BA, Mayne L, Rumbley J, Englander SW, Sosnick TR. Fast and slow intermediate accumulation and the initial barrier mechanism in protein folding. *J. Mol. Biol.* 2002; 324:359–371. [PubMed: 12441113]
27. Mukherjee S, Chowdhury P, Bunagan MR, Gai F. Folding kinetics of a naturally occurring helical peptide: implication of the folding speed limit of helical proteins. *J. Phys. Chem. B.* 2008; 112:9146–9150. [PubMed: 18610960]
28. Hubner IA, Deeds EJ, Shakhnovich EI. Understanding ensemble protein folding at atomic detail. *Proc Natl Acad Sci U S A.* 2006; 103:17747–17752. [PubMed: 17095606]
29. Singhal N, Snow CD, Pande VS. Using path sampling to build better Markovian state models: Predicting the folding rate and mechanism of a tryptophan zipper beta hairpin. *Journal of Chemical Physics.* 2004; 121:415–425. [PubMed: 15260562]
30. Gruebele M. Protein folding: the free energy surface. *Curr. Opin. Struct. Biol.* 2002; 12:161–168. [PubMed: 11959492]
31. Bryngelson JD, Onuchic JN, Socci ND, Wolynes PG. Funnels, pathways, and the energy landscape of protein folding: a synthesis. *Proteins.* 1995; 21:167–195. [PubMed: 7784423]

32. Jacob M, Schmid FX. Protein folding as a diffusional process. *Biochemistry*. 1999; 38:13773–13779. [PubMed: 10529221]
33. Schuler B, Lipman EA, Eaton WA. Probing the free-energy surface for protein folding with single-molecule fluorescence spectroscopy. *Nature*. 2002; 419:743–747. [PubMed: 12384704]
34. Crane JC, Koepf EK, Kelly JW, Gruebele M. Mapping the transition state of the WW domain beta-sheet. *J Mol Biol*. 2000; 298:283–292. [PubMed: 10764597]
35. Sato S, Religa TL, Daggett V, Fersht AR. Testing protein-folding simulations by experiment: B domain of protein A. *Proc. Natl. Acad. Sci. U S A*. 2004; 101:6952–6956. [PubMed: 15069202]
36. Burdette SC, Walkup GK, Spingler B, Tsien RY, Lippard SJ. Fluorescent sensors for Zn(2+) based on a fluorescein platform: synthesis, properties and intracellular distribution. *J Am Chem Soc*. 2001; 123:7831–7841. [PubMed: 11493056]
37. Woodrooffe CC, Masalha R, Barnes KR, Frederickson CJ, Lippard SJ. Membrane-permeable and -impermeable sensors of the Zinpyr family and their application to imaging of hippocampal zinc in vivo. *Chem Biol*. 2004; 11:1659–1666. [PubMed: 15610850]
38. Jackson AP, Timmerman MP, Bagshaw CR, Ashley CC. The kinetics of calcium binding to fura-2 and indo-1. *FEBS Lett*. 1987; 216:35–39. [PubMed: 3108033]
39. Vijay-Kumar S, Bugg CE, Wilkinson KD, Vierstra RD, Hatfield PM, Cook WJ. Comparison of the three-dimensional structures of human, yeast, and oat ubiquitin. *J. Biol. Chem*. 1987; 262:6396–6399. [PubMed: 3032965]

Scheme 1.

Figure 1. BiHis sites and protein folding

a) Folding reaction surface in the absence (black) and presence of M(etal) ions (red). **b)** The structure of Ubiquitin (1UBQ) (39) is shown with the locations of site *h* (across sheets β 3- β 4) and site *k* (at the N-terminus of the α -helix) indicated by the grey circles. Each bi-Histidine site is individually introduced into the protein. **c)** The proposed thermodynamic cycle for metal binding. The folding rate in the absence of metal ion is given by $k_f = K_{eq}^{U-I} k_{trans}$ while the change in equilibrium stability and folding rate as a function of metal are given by Eqs. 1 & 2.

Fig 2. Zinc-specific fluorophore, Zinpyr-1

a) The chemical structure and **b)** excitation and emission spectra of ZP-1.

Fig. 3. Models for metal-dependent changes in protein stability

Protein stability as a function of $[\text{Zn}^{2+}]$ is obtained from the changes in the k_f and k_u , for site *h* and site *k* on Ub and a helical site on Protein A (Y15H, Q11H). The change in stability is fit to a model allowing only for native state binding (dashed line) and a linked equilibrium binding model allowing for both native and denatured state binding (solid line). Values are listed in Table 1.

Fig. 4. Metal Binding is in Fast Equilibrium

For Ub, the equilibrium stability determined by equilibrium denaturant profiles (&066D;) agree with the values obtained from the changes in the k_f and k_u (o) according to Eq. 2.

Insets: Equilibrium denaturant melts for each site as measured by CD at 226 ± 5 nm normalized to the value of the native and denatured states, as a function of $[Zn^{2+}]$ (values in μM). The measurements are conducted in 50 mM HEPES, 100 mM NaCl, pH 7.5.

Fig. 5. BiHis binding parameters obtained from equilibrium competition measurements
 Observed Zn^{2+} :ZP-1 binding rates, obtained from the change in the dye's fluorescence signal, are obtained when 3 μM ZP-1 (final concentration) is mixed with increasing concentrations of biHis variants of Ub or BdpA that have been pre-equilibrated with 1 μM Zn^{2+} (final concentration). The biHis sites on the proteins reduce $[\text{Zn}^{2+}]_{\text{free}}$, which reduces the observed rate of ZP: Zn^{2+} complex formation according to fluorescence emission of ZP-1 above 520 nm. The binding constants are determined from Eq. 4.

Fig. 6. Kinetic competition between Zn^{2+} binding to either ZP-1 or biHis sites on the protein in either the folded or unfolded state

The rate of ZP-1 dye binding decreases when $3\ \mu\text{M}$ ZP-1, $1\ \mu\text{M}$ Zn^{2+} (final concentrations) and increasing concentrations of Ub are simultaneously mixed. The α -helical mutant site k is either folded (0 or 1.5 M GdmCl) or unfolded (6 M GdmCl), and each trace represents a different Ub concentration. The effect of the β -sheet mutant site h in 6M GdmCl is shown as well. The range of Ub concentrations used is 33-200 and $0.2\text{-}15\ \mu\text{M}$ for the β -sheet site- h and the α -helical site- k , respectively. The lines are global fits of the data using the program DynaFit (21).

Figure 7. Proposed Ub folding pathway placed on a one-dimension free energy reaction surface
 The transition state ensemble (TSE) has a minimal obligate core consisting of the carboxy-terminus of α -helix and four aligned β -strands, as defined by biHis sites having $\psi=1$. Around this core, biHis sites, including sites *h* and *k* studied here, have intermediate ψ -values. These values are indicative of sites which are fractionally formed or distorted. The expanded portion highlights the “pre-TSE”, which is the thermodynamic well prior to the true $P_{1/2}$ position on the landscape where half the molecules successfully fold to the native state. In the pre-TSE, some of the fractional sites are depicted as additional structure formed in fast equilibrium. From this state, molecules either form more structure and progress forward over the highest point on the reaction surface according to k_{trans} , or lose structure and return back towards the unfolded state according to k_{back} . The overall rate of folding is described as $k_f = K_{eq}^{U-I} k_{trans}$, where K_{eq}^{U-I} is the equilibrium constant for the pre-TSE well.

Table 1

Summary of biHis Binding Constants¹

Measurement	Ub site <i>h</i> β -sheet (R42H, V70H)		Ub site <i>k</i> α -helix (E24H, A28H)		Protein A, α -helix Q11H/Y15H,N29H/Q33H ³	
	$K_{eq}^{S,N}$	$K_{eq}^{S,D}$	$K_{eq}^{S,N}$	$K_{eq}^{S,D}$	$K_{eq}^{S,N}$	$K_{eq}^{S,D}$
Change in equilibrium stability	32 $\pm$ 3	294 $\pm$ 73	3.2 $\pm$ 0.2	85 $\pm$ 13	16 $\pm$ 1	65 $\pm$ 6
Equilibrium Competition	> 55 μ M ²	ND	1.8 $\pm$ 0.2 (1.5 M)	ND	3.2 $\pm$ 0.4 (2.2 M)	ND
Kinetic Competition	ND ²	> 240 μ M	2.5 $\pm$ 1.3 (1.5 M)	118.00 $\pm$ 0.02 (6 M)	ND	ND

¹The binding constant for β -sheet site *h* and α -helical site *k* under folding or unfolding conditions are listed for three types of experiments: Equilibrium stability derived from kinetic folding studies (Fig. 3), Equilibrium Competition (EqComp, Fig. 5) and Kinetic Competition (KinComp, Fig. 6) assays. Values in parenthesis are the GdmCl concentrations where the measurements are conducted. ND = not determined. Units are μ M. The statistical errors are absent for the KinComp measurements as they are not generated by the output of the global fit using Dynafit; the statistical errors for Ub site *k* are obtained from repeated measurements (see Table 2).

²The binding of Zn^{2+} was indistinguishable from control experiments performed using a Ub variant lacking a biHis site

³Change in equilibrium stability was measured with the Q11H/Y15H variant while the EqComp assays used the N29H/Q33H biHis variant of Protein A

Table 2Kinetic Competition Assays¹

[GdmCl]	Ub Site k α -helix (E24H, A28H)		
	k_{on}^{biHis}	k_{off}^{biHis}	K_{eq}^{biHis}
0	7	92	15
1.5	27 $\pm$ 22 (52, 20, 10)	76 $\pm$ 11 (88, 74, 66)	2.8 $\pm$ 2.3
6	4	471	118

¹ The values of the on- and off-rate constants derived from global fits of five independent experiments at different protein concentrations (See Figs. 6,7). The binding constant was calculated according to $K_{eq}^{biHis} = k_{off}^{biHis} / k_{on}^{biHis}$. The statistical errors generally are absent as they not generated by the output of the global fit using Dynafit; the statistical errors listed are obtained from repeated measurements (values in parentheses listed underneath). [GdmCl] is given in units of M. Units of k_{on}^{biHis} , k_{off}^{Ub} and K_{eq}^S are $(\mu\text{M})^{-1} \text{ s}^{-1}$, s^{-1} , and μM , respectively.