Protein conformer selection by ligand binding observed with crystallography

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Abstract

A large-scale movement between “closed” and “open” conformations of a protein loop was observed directly with protein crystallography by trapping individual conformers through binding of an exogenous ligand and characterization with solution kinetics. The buried indole ring of Trp¹⁹¹ in cytochrome *c* peroxidase (CCP) was displaced by exogenous ligands, causing a conformational change of loop Pro¹⁹⁰–Asn¹⁹⁵ and exposing Trp¹⁹¹ to the protein surface. Kinetic measurements are consistent with a two-step binding mechanism in which the rate-limiting step is a transition of the protein to the open state, which then binds the ligand. This large-scale conformational change of a functionally important region of CCP is independent of ligand and indicates that about 4% of the wild-type protein is in the open form in solution at any given time.

Keywords: artificial cavity; cytochrome *c* peroxidase; kinetics; ligand binding; loop movement; protein crystallography; site-directed mutagenesis

Much evidence exists that proteins exhibit considerable flexibility. Ligands are often found in the center of proteins with no observable path to the surface, suggesting that the protein must transiently unfold in order for the ligand to enter. Examples include myoglobin and hemoglobin oxygen binding to the heme (Karplus & Petsko, 1990), fatty acid binding to fatty acid binding protein (LaLonde et al., 1994), retinol binding to retinol binding protein (Zanotti et al., 1993), and ligand binding to artificial cavities introduced into proteins (Eriksson et al., 1992; Fitzgerald et al., 1994, 1996; Feher et al., 1996). Flexible surface-loops in protein structure are thought to play an important role in substrate binding and molecular recognition. Crystal structures have shown that, for a number of enzymes and antibodies, substrate and/or antigen binding causes a conformational change of a surface-loop either from an “open” to a “closed” form to partially cover the bound substrate (Joseph et al., 1990; Lolis & Petsko, 1990; Derewenda et al., 1992; Jia et al., 1995), or from a “partially closed” to “open” form, resulting in an induced fit for the antigen or ligand binding (Rini et al., 1992; Concha et al., 1993). These conformational changes have been observed directly by NMR, and the relevance of these motions to dihydrofolate reductase function has been demonstrated (Epstein et al., 1995). All of these examples reveal that

proteins switch between conformations and that these changes are often key to protein function.

Here we trap a large conformational change of a protein loop by binding an exogenous ligand to the protein and directly visualizing the structure by X-ray crystallography. The ligand displaces a buried tryptophan side chain and leaves the displaced loop on the surface. This loop displacement was first observed in the crystal structure of the F202G (i.e., Phe²⁰² replaced by Gly) mutant of cytochrome *c* peroxidase, which is somewhat less stable relative to wild-type. The same loop displacement was observed subsequently in the wild-type protein. Kinetic measurements are consistent with a model in which the rate-limiting step of ligand binding is a first-order reaction of the protein, presumably, the protein unfolding from the closed to the open state, followed by relatively rapid ligand binding. In the absence of ligands, as calculated from solution kinetics, the ratio of the open to closed conformer is on the order of 1:28. In the F202G mutant, the protein is more flexible and the ratio of loop open to closed forms is 1:11.

Results

Crystal structures of ligand trapped conformational changes

A buried cavity was introduced into CCP by replacing Phe²⁰² with glycine (F202G). The crystal structure (Table 1) showed that a cavity (cavity 202) was formed and, other than the fact that the *B*-values for many of the loops in the protein had increased, the structure was otherwise largely undisturbed. Several cationic ligands, such as the protonated forms of 1,2-dimethylimidazole and

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Abbreviations: CCP, cytochrome *c* peroxidase; wtCCP, wild-type CCP; cyt *c*, cytochrome *c*; DMI⁺, 1,2-dimethylimidazolium; 2a5mT⁺, 2-amino-5-methylthiazolium; MPD, 2-methyl-2,4-pentanediol.

Table 1. X-ray diffraction data collection and refinement statistics

	F202G ^a	F202G ^b soaked in DMI ^c	wt CCP ^b soaked in 2a5mT ^d	F202G ^b soaked in 2a5mT ^d	F202G ^b soaked out 2a5mT ^d
Data collection					
Crystal form	Yeast	Yeast	MKT	Yeast	Yeast
Unit cell					
<i>a</i> (Å)	108.3	107.7	105.1	107.5	107.9
<i>b</i> (Å)	77.1	76.6	74.2	76.1	76.6
<i>c</i> (Å)	51.7	51.7	45.2	51.6	51.8
Resolution (Å)	2.1	2.4	2.0	2.0	2.0
<i>I</i> / σ _{<i>I</i>} (av)	20.6	6.5	30.9	22.3	14.9
<i>I</i> / σ _{<i>I</i>} (last shell)	1.8	1.0	3.6	1.6	1.7
No. of reflections	23,195	25,453	19,829	27,395	27,261
Completeness (%)	82	98	82	93	94
<i>R</i> _{sym} ^e	0.054	0.097	0.049	0.068	0.070
Refinement					
<i>R</i>	0.18	0.18	0.20	0.20	0.21
Resolution (Å)	2.1	2.4	2.0	2.0	2.0
No. of waters	98	98	98	98	98

^aThe crystal was grown by dialysis against distilled water (PDB code 1CCJ).

^bCrystals were grown and preserved in MPD.

^cStructure refined including DMI (PDB code 1CCI).

^dStructures were refined with loop 190–195 omitted.

^e*R*_{sym} represents the agreement between *I*_o for symmetry equivalent reflections. *I*/ σ _{*I*} (last shell) is the average *I*/ σ _{*I*} for the 10% of the data at highest resolution. *R* values give the crystallographic residuals between the observed and model-derived structure factor amplitudes. Both F202G and wtCCP were soaked in 50 mM DMI⁺ or 50 mM 2a5mT⁺.

aniline, which were found previously to bind in the W191G mutant cavity (Fitzgerald et al., 1994, 1996; R.A. Musah, M.M. Fitzgerald, D.E. McRee, & D.B. Goodin, unpubl. results), also bind at the position of the Trp¹⁹¹ side chain (named “191 cavity” or “191

position”) of F202G. This binding is accompanied by rearrangement of loop 190–195 and displacement of the Trp¹⁹¹ side chain. Similar to a rearrangement seen previously for W191C (Fitzgerald et al., 1996). After soaking a crystal of F202G in 50 mM

DMI⁺, the $2F_o - F_c$ map (Fig. 1) showed that there was no electron density at the position of the main chain between residues 190 and 195. Instead, a new, continuous electron density, beginning at Pro¹⁹⁰ and ending at Asn¹⁹⁵, had appeared in what was formerly solvent. The electron density for the side chain of Trp¹⁹¹ at the protein surface was clear and could be well fitted. After refinement of the model fit to the new loop density and with DMI⁺ at the 191 cavity, the R -factor at 2.4 Å was 17.8% (Table 1). The maximal displacement of this loop was 8.7 Å for the alpha carbon (Trp¹⁹¹-C α) and 12.6 Å for the side chain (Trp¹⁹¹-CD1). Pro¹⁹⁰ changes from *trans* to *cis*, forming one hinge point, and Asn¹⁹⁵ interchanges with the side-chain and main-chain positions at the other end to form the second hinge. The value of the pseudo dihedral angle of Trp¹⁹¹(C α_{closed})-Gly¹⁸⁹(C α_{closed})-Asn¹⁹⁵(C α_{open})-Trp¹⁹¹(C α_{open}) is 107° from the closed to the open conformer about an axis passing through two hinge points, Gly¹⁸⁹-C and Asn¹⁹⁵-C. In the loop open form, the hydrophobic side chain of Trp¹⁹¹ on the protein surface was not stabilized by any crystal contact or hydrogen bond.

Loop opening was also observed in wild-type CCP crystals when they were soaked in 2-amino-5-methylthiazolium iodide (Table 1; Fig. 2). Of all ligands found thus far bound in the W191G cavity, 2a5mT⁺ has the highest affinity (R.A. Musah & D.B. Goodin, unpubl. results). For this reason, 2a5mT⁺ was expected to strongly compete with the indole side chain, and the sulfur atom of 2a5mT⁺ would be expected to show a greater density difference over the C and N atoms of the Trp¹⁹¹ indole side chain. After soaking a wtCCP crystal in 50 mM 2a5mT⁺, the position of the sulfur atom was indicated by a positive density peak at $>5\sigma$ in the $F_o - F_c$ map (Fig. 2). By comparison to the earlier F202G loop open structure, it was observed that the electron density of loop 190–195 represents a mixture of open and closed forms. A series of extrapolated $(F_{\text{soaked}} - F_{\text{unsoaked}})/(\text{partial fractional occupancy})$ difference maps assuming 0.10, 0.20, and 0.30 partial fractional occupancy were calculated to estimate the partial occupancy of the open form of the loop and to make a map that shows only the open form. An

advantage of this method is that it is done in the absence of any model(s) and thus eliminates model bias. In the 0.20 partial occupancy map (i.e., with the differences scaled by a factor of 5), the map showed a fully occupied loop, no density at the main chain of the closed position, and strong density at the ligand binding site where the indole side chain had been displaced (Fig. 3). Using this method, the fractional occupancy of the closed and open forms of the loop were estimated to be 0.8 and 0.2, respectively.

In addition, we also refined the loop occupancy using a model containing both the loop in and loop out partial structures. The relative occupancies were refined in four groups. Group 1 contained the Pro¹⁹⁰ hinge, group 2 contained the loop residues 191–194, except for the indole side chain of 191, which was placed in group 3, and group 4 contained the Asn¹⁹⁵ hinge. The indole side chain was placed in a separate group because overlap of its density with that of 2a5mT⁺ would have resulted in an overestimation of the closed form. The refinement was done in two ways. In the first, the B -values were fixed at 20.0, and the relative occupancies of the two slit parts were allowed to refine. This led to an occupancy of 0.62 for the loop closed chain and a corresponding occupancy of 0.38 for the loop open chain. In the second, the B -values were freed and allowed to change. In this case, the occupancies refined to 0.61 and 0.39, respectively. These observations were further verified by the observation of convergence (albeit slow) after 100 cycles of refinement when the starting occupancy ratios were at 0.8 open and 0.5. Refinement from above or below in occupancy eventually gave essentially the same values. Thus, in this case, we found that the extrapolated difference method may not be entirely reliable for estimating the value of the partial structure occupancy of the loop. However, it is useful in map interpretation because it cleanly separates the density for the two interleaved structures from one other, and simplifies model-building and interpretation.

The opening of loop 190–195 is a reversible process in the CCP crystal. After soaking an F202G crystal in 50 mM 2a5mT⁺ at pH 5.2 for 15 min, the loop 190–195 was found partially open. Two 2a5mT⁺ molecules were found in the F202G structure: one at

Fig. 2. Open loop conformer partially trapped by binding of ligand to wtCCP. $2F_o - F_c$ electron density map (1σ , gray lines) of wtCCP soaked with 2a5mT⁺ indicates that part of the loop 190–195 is in a mixture of open and closed conformers because there is density at both positions (closed model, thin lines, PDB code 1CCA; open model, thick lines, PDB code 1CCI). The $F_o - F_c$ difference map (black, thick contours) is contoured at $+5\sigma$ and shows a peak at the position of the known 2a5mT⁺ binding site (R.A. Musah & D.B. Goodin, unpubl. results). The position of 2a5mT⁺ density is indicated, but a model is not shown. The crystal was soaked in 50 mM 2a5mT⁺, pH 5.2, for 20 min. The F_c and α_c of the wtCCP (Fitzgerald et al., 1994) with residues 189–195 omitted were used to construct the $2F_o - F_c$ and $F_o - F_c$ maps.

Fig. 3. Open loop conformer extracted from open/closed mixture in Figure 2 by extrapolation. Extrapolated F map (gray lines) assuming 20% partial occupancy is shown superimposed on the structure of the open loop form (thick lines) and closed loop model (thin lines). The map shows only density at the open form and at the position of the $2a5mT^+$ ligand (labeled $2a5mt$ in figure) with no density at the closed position (thin lines). The phases of the wtCCP (Fitzgerald et al., 1994) with residues 189–195 omitted were used to construct the $2F_{\text{extrapolated}} - F_{\text{wtCCP}}$ map (see Materials and methods).

Fig. 4. Superposition of backbone structures of the loop 190–195 closed conformer (orange, with closed loop in pink) and the open conformer (yellow, with open loop in white) of CCP. In the loop closed conformer, the Trp^{191} side chain (pink, ball-and-stick) is buried and forms an H-bond to Asp^{235} at the active site. In the loop open conformer, Trp^{191} (white, ball-and-stick) moves to the protein surface and becomes exposed to the solvent, leaving an empty binding pocket to which DMI^+ and $2a5mT^+$ bind.

the 191 position, with its sulfur atom position indicated by a positive density at 6σ in the difference map, and the second one at the 202 cavity, with its sulfur atom position indicated by a positive density at 5σ (Table 1; figure not shown). After data collection was complete, the F202G crystal was then transferred into distilled water and soaked for 2 h to remove the $2a5mT^+$. The $2F_o - F_c$ omit map (Table 1; figure not shown) showed that, after washing $2a5mT^+$ out of the crystal, the open loop moved back to the closed form, demonstrating that the loop opening is a reversible process in the F202G crystal.

Kinetics of conformer change

The kinetics of ligand binding can be rationalized in terms of Equation 1, where E_c and E_o represent the closed and open conformers of the protein, respectively, and E_oL is ligand-bound protein. Both the apparent rate (k_{obs}) and the dissociation constant (K_{d3}) of binding of DMI^+ to F202G and wtCCP were determined by measuring the absorption changes in the Soret band. Titration of both wtCCP and F202G with a high concentration of DMI^+ (>50 mM) resulted in a red shift of the heme Soret band from 408 nm to 416 nm, which was accompanied by an increase in the intensities of the β and α bands at 530 and 560 nm, respectively. The observed spectral changes are consistent with a high-spin to the low-spin state transition of the ferric iron-heme complex (Smulevich et al., 1991; Goodin & McRee, 1993). The kinetics of these spectral shifts were nearly exponential and extremely slow, with lifetimes of 10^3 for F202G and 10^4 for wtCCP at 20°C . Because a similar low-spin spectrum has been observed previously for the W191G mutant with DMI^+ bound in the 191 cavity, the red shift observed during titration of F202G and wild-type with DMI^+ was rationalized in terms of the displacement of Trp 191 with DMI^+ , accompanied by loop movement as had been observed in the crystal structures. To determine whether the red shift was due to non-specific effects at high concentration, two neutral ligands, 2-mercaptoimidazole and thiazole, which were not expected to bind in the 191 cavity, were added to the wild-type CCP solution to give a final concentration of 200 mM and 133 mM, respectively. After equilibration for 12 h at room temperature ($\sim 22^\circ\text{C}$), no red shift was observed. Figure 5 shows the transition of F202G from high spin to low spin upon addition of DMI^+ . The K_d at 20°C , as determined from a Scatchard plot (Fig. 5, inset), is 20 mM for binding of DMI^+ to F202G, and approximately 50 mM for the binding of DMI^+ to wtCCP.

The kinetics of ligand binding as a function of ligand concentration (Fig. 6), are consistent with a pre-equilibrium model (Equation 1) under conditions in which the second step (ligand-binding) (K_{d2}) is much faster than a first zero-order pre-equilibration step (K_{d1}) (Fersht, 1984). Fitting the data in Figure 6 to a pre-steady state kinetic model (Equation 2, see Materials and methods), gave estimates for K_{d1} of approximately $11.4 (\pm 0.3)$ for F202G and $28 (\pm 6)$ for wtCCP.

Fig. 5. Dissociation constant determination for binding of DMI^+ to F202G. UV-vis absorption spectra obtained by titration of F202G with DMI^+ , pH 6. Inset: Scatchard plot utilized to obtain K_{d3} from the difference absorbance (ΔAbs) at 418 nm. The fraction bound (ν) was determined by extrapolating $1/\Delta\text{Abs}$ versus $1/[DMI^+]$ to infinite DMI^+ concentration assuming one binding site (Fitzgerald et al., 1994).

In the mechanism in Equation 1, the observed induced-fit binding (upper path, loop opening simultaneous with ligand binding) is broken into two steps along the lower path: (1) loop opening independent of ligand, and (2) lock-and-key binding in which the ligand binds to the loop open conformer.

Discussion

Implications for protein flexibility

Our experiments show that the open and closed forms of wtCCP are in constant equilibrium in solution even in the absence of

Fig. 6. Solution kinetics of DMI^+ binding to wtCCP consistent with a pre-equilibrium of the protein. The apparent rate (k_{obs}) of binding of DMI^+ to wtCCP is plotted as a function of DMI^+ concentration $[L]$. The raw data (circles) were fitted to a line using Equation 2 in Materials and methods. The shape of the curve is characteristic of a process with a pre-equilibrium first-order step followed by a relatively fast second-order binding of ligand where the apparent rate of binding decreases with increasing ligand concentration.

ligand. The loop open conformer in the native form of CCP leaves an empty binding pocket (filled with water molecules) to which DMI⁺ can bind. The binding of DMI⁺ selects this open conformer and shifts the loop close–open equilibrium toward the loop open conformer. We estimate that, at any given time, about 4% of the wild-type protein and about 9% of the destabilized F202G mutant are in the loop open conformation. In the absence of ligand binding, it would be difficult to observe this motion because it represents such a small percentage of the total population. It can be surmised that other loops in the protein may also be in similar motions that are not detectable due to the lack of a means of trapping or observing them. The binding of ligand in our case does not “cause” the loop to open, but, rather, because the ligand binds to the open conformer, it selects this conformer and shifts the equilibrium to the open conformer. This binding only appears to be induced fit because of the equilibrium shift; before ligand addition, a small amount of the protein pre-exists in the open conformer as shown by the pre-equilibration kinetics.

This mechanism has also been observed for the motion of loop-6 in triosephosphate isomerase (Williams & McDemott, 1995). The loop motion is not ligand gated, but a natural motion of the protein as revealed by NMR studies, and the population ratio between loop open and closed forms, is shifted by the presence of ligand: it changes from 10:1 (without ligand bound) to 1:10 (with ligand bound). NMR data (Williams & McDemott, 1995) reveal that the loop open and closed conformers are in equilibrium in the native protein, and binding of the substrate shifts this equilibrium toward the loop closed conformer.

Is the loop opening involved in the electron transfer process?

CCP catalyzes the transfer of electrons from reduced cyt *c* to peroxide, forming water. In the oxidized “ES” state, Trp¹⁹¹ is reversibly oxidized to a stable cation free radical, which is believed to play a major role in cyt *c* oxidation (Erman et al., 1989; Sivaraja et al., 1989; Houseman et al., 1993; Huyett et al., 1995). Although the details of how electrons are transferred from reduced cyt *c* to CCP are still unclear, the crystal structure of the CCP–cytochrome *c* complex does indicate that cyt *c* binds near the loop 190–195 region of CCP (Pelletier & Kraut, 1992). The significantly increased thermodynamic motion of loop 190–195 was not only found in the F202G mutant protein, but also in a previously reported structure of nitric oxide (NO) bound to the heme Fe of wtCCP, where a slight movement of Trp¹⁹¹ away from His¹⁷⁵ and an increase in the *B*-factor of Trp¹⁹¹ from 18.7 Å² to 28.3 Å² was observed (Edwards et al., 1988; Edwards & Poulos, 1990). This experiment reveals that the binding of NO to the distal side of the heme iron, the same site where peroxide binds, can specifically affect the thermodynamic motion of loop 190–195. This raises the interesting question of whether or not the loop opening is involved in the electron transfer process. Because Trp¹⁹¹ is the site of the free radical in the ES complex, loop opening would effectively transport the electron hole on the side-chain radical to the surface, where it could interact directly with an electron donor. However, there are a number of experimental results that disfavor this idea. (1) The loop opening kinetics (calculated half-life $\gg 10^2$ s from Equation 2 in Materials and methods) appear to be too slow for the electron transfer. (2) There are no reported structures of the ES form (Edwards et al., 1987; Fulop et al., 1994; Miller et al., 1994), indicating that the *B*-factor of loop 190–195 is increased. (3) The structure of the CCP–cyt *c* complex (Pelletier & Kraut, 1992) does

not show the *B*-factor of loop 190–195 to be significantly altered. There is, however, no direct evidence to exclude this possibility.

Materials and methods

Materials

The F202G mutant of CCP was constructed using site-directed mutagenesis as described previously, and both wild-type and F202G protein were expressed and purified as described previously (Fitzgerald et al., 1994).

X-ray crystallography

Structural determination was done as described earlier (Fitzgerald et al., 1994). Single crystals of X-ray diffraction quality were grown from 25% MPD by vapor diffusion (Wang et al., 1990). F202G was crystallized in sitting drops with approximately 0.17 mM CCP, 70–110 mM potassium phosphate, pH 6, and 8.3% MPD against 25% MPD. Large (0.8 × 0.4 × 0.03 mm) F202G crystals were obtained by macroseeding small F202G crystal seeds (~0.2 mm in length) into freshly prepared protein solution (0.17 mM CCP in 80 mM KPO₄ and 8.3% MPD). WtCCP and F202G crystals were soaked for about 45 min in the test compounds before mounting (50 mM DMI in water, pH adjusted to 6.0 with H₃PO₄, or 50 mM 2a5mT in water, pH adjusted to 5.2 with H₃PO₄). X-ray diffraction data were collected at 17 °C using Cu K α radiation from the rotating anode of Rigaku X-ray generator and a Siemens area detector. Data were analyzed by difference Fourier techniques using the XtalView software (McRee, 1993). Difference Fourier maps ($|F_{o,F202G}| - |F_{c,wtCCP}|$, $\alpha_{c,wtCCP}$ or $|F_{o,soaked}| - |F_{c,unsoaked}|$, $\alpha_{c,unsoaked}$) were used for detecting structural changes. Models were built using XtalView and refined with repeated cycles of positional and *B*-factor refinements using the program X-PLOR (Brünger et al., 1987). Partial occupancy refinements of the loop closed/open model were done using SHELX-97 (George Sheldrick, University of Göttingen, Germany).

Extrapolated difference maps were calculated using the equation $F_{\text{extrapolated}} = F_{o,wtCCP} + (F_{o,F202G} - F_{o,wtCCP})/(\text{partial occupancy})$ (Genick et al., 1997). Outliers were rejected by not using reflections where $|F_{o,F202G} - F_{o,wtCCP}|/F_{o,wtCCP} > 0.4$. Phases with the loop 190–195 omitted from the wild-type model were used and the partial occupancy was set to 0.2 for the map used in Figure 3.

Kinetics

Substitution of DMI⁺ for the indole ring of Trp¹⁹¹ and opening of loop 190–195 shifted the UV-vis absorption spectrum of CCP from high spin ($\lambda_{\text{max}} = 408$ nm) to low spin ($\lambda_{\text{max}} = 418$ nm). This λ_{max} shift was used to measure the apparent rate of binding of DMI⁺ to CCP (k_{obs}) and the dissociation constant (K_{d3} , Equation 1). For simplicity, because 2a5mT⁺ binds to two sites in F202G, whereas DMI⁺ binds to only one, only DMI⁺ dissociation constants were measured and compared. Compounds were obtained from Sigma or Aldrich. UV-vis spectra were collected at 20 °C using a Hewlett-Packard 8452A or 8453 diode-array spectrophotometer.

Binding assays were performed by difference absorption spectroscopy (Fitzgerald et al., 1994). Stock solutions of 1.0 M dimethylimidazole were prepared with 100 mM Bis-tris propane/

MES, pH 6.0. Stock protein solutions were prepared by dissolving CCP crystals in 500 mM Bis-tris propane/MES, pH 6.0. This stock solution was then diluted into the stated buffer, so as to give an absorbance of 1.0 at the Soret maximum. The protein solution was allowed to equilibrate at 20 °C for 20 min in the spectrophotometer with stirring, and the instrument was blanked. An aliquot of the DMI⁺ stock solution (10 µL for F202G and 100 µL for wtCCP) was added to the cuvette, and the change in absorption spectrum was recorded after a 30-min equilibration time. Dissociation constants were determined from Scatchard plots based on the difference absorbance at the Soret maximum of the heme. Because of the extremely long equilibration time during the titration (30 min per addition for a total of 7 h for F202G, and 12 h for each addition for a total of 48 h for wtCCP) at 20 °C, the protein was subject to significant unfolding. To account for the increased light scattering due to the protein unfolding, the ratio of 422 nm to 408 nm was used to calculate the concentration used in the Scatchard plot.

Apparent rates (k_{obs}) of binding of DMI⁺ to F202G or wtCCP were measured at 418 nm. The data were fitted to a single exponential curve that gave the value of k_{obs} , and a straight line background drift. The equation $k_{obs} = k_1 + k_{-1}/([L]/K_{d2} + 1)$ (Equation 4.52 in Fersht, 1984) describes a pre-equilibrium model (Equation 1) under conditions in which the second step ($K_{d2} = k_{-2}/k_2$) is much faster than the first ($K_{d1} = k_{-1}/k_1$). Because we have experimental data for $K_{d3} = k_{-3}/k_3$ in Equation 1, K_{d2} in the above equation can be replaced by $K_{d2} = K_{d3}/K_{d1} = K_{d3} * k_1/k_{-1}$, which gives Equation 2:

$$k_{obs} = k_1 + k_{-1}/\{([L] * k_{-1})/(K_{d3} * k_1) + 1\}. \quad (2)$$

The experimental data were plotted as k_{obs} versus $[L]$ in Figure 5. Fitting this plot using Equation 2 gives k_1 and k_{-1} , and, therefore, K_{d1} . This fitting indicates that step 1 in Equation 1 is rate limiting. The program Sigma Plot (Jandel Scientific) was used for fitting operations.

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