Guanidinium derivatives bind preferentially and trigger long-distance conformational changes in an engineered T4 lysozyme

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(RECEIVED December 7, 2005; FINAL REVISION January 17, 2006; ACCEPTED January 17, 2006)

Abstract

The binding of guanidinium ion has been shown to promote a large-scale translation of a tandemly duplicated helix in an engineered mutant of T4 lysozyme. The guanidinium ion acts as a surrogate for the guanidino group of an arginine side chain. Here we determine whether methyl- and ethylguanidinium provide better mimics. The results show that addition of the hydrophobic moieties to the ligand enhances the binding affinity concomitant with reduction in ligand solubility. Crystallographic analysis confirms that binding of the alternative ligands to the engineered site still drives the large-scale conformational change. Thermal analysis and NMR data show, in comparison to guanidinium, an increase in protein stability and in ligand affinity. This is presumably due to the successive increase in hydrophobicity in going from guanidinium to ethylguanidinium. A fluorescence-based optical method was developed to sense the ligand-triggered helix translation in solution. The results are a first step in the de novo design of a molecular switch that is not related to the normal function of the protein.

Keywords: sequence duplication; T4 lysozyme; conformational change; arginine; guanidinium

The design of ligand-gated conformational changes in proteins for possible nano-biotechnological applications (Doi and Yanagawa 1999; Mizoue and Chazin 2002; Dwyer et al. 2003; Looger et al. 2003; Luo et al. 2003; Guntas and Ostermeier 2004; Joyce et al. 2004) is of general interest.

Most, if not all, of the switches reported to date make use of an intrinsic property of the protein in question (e.g., a ligand-induced hinge-bending motion, or a ligand driven disorder-to-order transition). We here describe a type of conformational change that has a large magnitude and could, in principle, be introduced into any protein of interest (i.e., it is not restricted to a host protein that undergoes large-scale changes as part of its biological function).

We recently designed a molecular switch in a T4 lysozyme construct that promotes a large-scale (~20 Å) translocation of an α -helix but is unrelated to the function of the protein (Yousef et al. 2004) (Fig. 1). The design was based in part on the use of a duplicated helical sequence (mutant L20; Sagermann et al. 1999) to allow large-scale change between two alternative conformational states (Sagermann et al. 2003), and in part on the

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Article and publication are at <http://www.proteinscience.org/cgi/doi/10.1110/ps.052020606>.

Figure 1. Use of a duplicated α -helix plus a point mutation to engineer large-scale conformational changes. The point mutation is highlighted (Arg63 to Ala in the L20/R63A mutant, corresponding to Arg52 to Ala in wild type). Shown at the *top* of the figure is the sequence of T4 lysozyme that includes the tandem repeat. Residues Asn₅₁–Ile₆₁ correspond to Helix B in the wild-type protein. Residues Asn₄₀–Ile₅₀ are a tandem duplicate. Shown *below* is a superposition of the alternative structures that can result from the use of this sequence repeat. The structures are arranged so that the duplicated helix is at the *bottom* of the figure with its C terminus at the *left*. When guanidinium ion binds at the ligand binding site it stabilizes the C terminus and the helix extends to the right (liganded L20/R63A structure shown in red). The structure of L20, which has an arginine at site 63, is essentially identical. In the absence of guanidinium ion the C-terminal region of the helix is destabilized and the helix extends to the left (nonliganded L20/R63A structure shown in blue). The position occupied by Ile39 in the respective structures is shown. Also shown are the locations of the NMR probe, Leu32, and Tyr24, the site of bimane binding.

identification of a critical arginine residue that stabilizes one state relative to the other (Yousef et al. 2004). Mutating this arginine to alanine (mutant L20/R63A) caused the helix to shift ~ 20 Å and to partially unravel. Upon addition of guanidinium ion, a surrogate for the guanidino head-group of the arginine side chain, the helix returned to its original conformation. Thus, the ligand controls a shift between the two conformations spanning over 20 Å.

The idea of using a guanidinium ion to drive the conformational changes was based on its similarity to the side chain of Arg63. In order to mimic the side chain more closely we have investigated the effectiveness of methyl- and ethylguanidinium. These ligands permit similar hydrogen bonding but have different hydrophobic characteristics. It has also been shown that increased alkyl chain length of guanidinium derivatives can have a profound effect on binding affinity (David et al. 1992). The role of hydrophobicity in ligand binding (Barratt et al. 2005; Boresch et al. 2005; Gratzer et al. 2005; Volkman et al. 2005; Weng et al. 2005) and in allosteric reactions (Hansch et al. 2003; Mekapati et al. 2005) has been investigated in a number of different systems.

We also developed an optical probe for the dynamic monitoring of ligand-dependent conformational changes. The design of the probe exploited a method that was previously used for mapping proximity within proteins based on the distance-dependent quenching of the fluorescent label bimane by tryptophan (Mansoor et al. 2002; Mansoor and Farrens 2004). We have optimized the method for one ligand (methylguanidinium) and found changes in fluorescence intensity consistent with the long-range motion in solution.

Results and Discussion

X-ray crystallography

The crystal structure of the “switch” mutant L20/R63A (see introduction) liganded to both methyl- and ethylguanidinium ions was determined at resolutions of 1.7 Å and 1.8 Å, respectively (see Materials and Methods). The resolution of the previously solved structure of the mutant liganded to guanidinium ion (Yousef et al. 2004)

was also extended from 2.0 Å to 1.45 Å. All three liganded structures were obtained in space group P3₂21, isomorphous with wild-type lysozyme and one of the two crystal forms of L20 (Sagermann et al. 1999). There is ample room for each of the ligands to bind without incurring possible contacts with neighboring molecules in the crystal. (The nonliganded L20/R63A structure is in a different space group.)

The X-ray structure determinations show that the binding of methyl- and ethylguanidinium drive the same large-scale conformational change as guanidinium ion (Fig. 2). The structures of the L20/R63A mutant with each of the three ligands are virtually identical, but differ radically from the ligand-free protein (Fig. 2). In all three cases the binding of the ligand promotes a conformational change that extends 20 Å or so from the site of binding.

The electron density difference map for methylguanidinium (Fig. 3B) clearly shows that the guanidinium portion occupies essentially the same position and makes the same hydrogen bonds as guanidinium ion itself (Fig. 3A). These interactions are identical to those made by the head group of Arg63 in the template protein L20 (Fig. 3D). The ligand binds in two different ways, however. In one conformation the methyl group occupies essentially the same position as C_δ of Arg63 and is 3.5 Å from C_β of Ala63. In the alternative position there is a rotation of ~180° about an axis in the plane of the ligand, which brings the methyl group within 3.3 Å of the carbonyl oxygen of Asn64 (Fig. 3B).

Similar alternative binding modes were observed when ethylguanidinium was bound (Fig. 3C). As before, the body of the ligand flips over in an ~180° rotation but the distal carbon has a distinct conformation not related by twofold symmetry. In one mode the proximal carbon

occupies a position analogous to C_δ of Arg63, but in neither mode does the distal carbon occupy the same position as C_γ of Arg63, as it would incur an unacceptable steric clash (Fig. 3, cf. C and D).

NMR spectroscopy

One way to map a ligand-binding site on a protein and to quantify the interaction strength is by exploiting changes in NMR chemical shifts upon ligand binding (Shuker et al. 1996; Hu et al. 2004; Azurmendi et al. 2005). The chemical shift reflects the local environment of a nuclear spin, and binding of a ligand nearby can change this environment. Heteronuclear single quantum coherence (HSQC) spectra are particularly useful for “NMR titration” studies and are usually conducted with uniformly ¹⁵N-labeled protein. The ¹H-¹⁵N HSQC technique cross-correlates the chemical shift of the ¹⁵N-nucleus of a nitrogen-containing group with the chemical shift of bound proton(s). In a growing number of cases, ligand binding and associated structural changes (if any) have been localized based on this technique (Clarkson and Campbell 2003; Maurer 2005).

The combined (¹H, ¹⁵N) chemical shifts in the presence of guanidinium ion are shown in Figure 4A. Residues with the largest shifts are within the N-terminal domain, the location of the duplicated helix (Fig. 1). Residues 75–90 (corresponding to 64–79 in wild type) are within the helix that connects the N- and C-terminal domains of the protein and the chemical shifts in this region might reflect some “hinge-bending” motion of the molecule as a whole associated with the translocation of the duplicated helix. The chemical shift perturbations increase as a function of ligand concentration. In the case of Leu32 the shifted peaks in the NMR spectra

Figure 2. Stereo pair showing that the backbone structures of L20/R63A lysozyme complexed with guanidinium ion (red), methylguanidinium (yellow), and ethylguanidinium (green) are virtually identical. The structure of L20/R63A in the absence of ligand (cyan) differs substantially.

Figure 3. Details of the stabilizing interactions of the loop at the C terminus of the engineered helix in the mutant L20/R63A in the presence of guanidinium (A), methylguanidinium (B), and ethylguanidinium (C). The superimposed $F_o - F_c$ difference maps contoured at 3σ (red) define the position of the ligands. The resolution of the maps is 1.45 Å, 1.7 Å, and 1.8 Å, respectively. The methylated and ethylated ligands adopt alternative conformations as shown. (D) Interactions made by Arg63 in the lysozyme (Molecule B, PDB code 262L). Similar interactions are made by Arg52 in wild-type lysozyme.

are free of overlap with neighboring peaks and, as such, are suitable for quantitative analysis. Leu32 is well ordered in both the liganded and unliganded crystal structures and is adjacent to the N terminus of the switch helix (Fig. 1). Hence it should be sensitive to confor-

mational rearrangement upon binding. For each ligand the chemical shift perturbation as a function of ligand concentration could be fit with a single binding site model (Fig. 4B–D). The dissociation constants and free energies of binding are given in Table 1. Notably, the most soluble ligand (guanidinium) has the lowest affinity, while the least soluble (ethylguanidinium) has the highest.

Thermal analysis

When Arg63 is truncated to Ala (in mutant L20/R63A), the stability of the protein is reduced by 6.1°C relative to L20 (Yousef et al. 2004). In high salt buffer similar to that used for crystallization, the melting temperature of L20/R63A is increased by 2.2°C in the presence of either 200 mM methylguanidinium or 200 mM ethylguanidinium ion (cf. 1.7°C in the presence of 200 mM guanidinium ion). Thermally promoted unfolding of T4 lysozymes is irreversible in this high salt buffer, making conversion of ΔT_m values into free energies problematical. Nevertheless, for these ligands the delay in the onset of irreversible unfolding is consistent with the binding constants determined via NMR in a more physiological salt; that is, as the ligands bind more tightly in low salt, the onset of irreversible unfolding is delayed to higher temperatures in 95% crystallization buffer.

Affinities of binding

The NMR binding data (Fig. 4; Table 1) show that the equilibrium binding constant for each ligand increases as the ligand becomes more hydrophobic. The thermal unfolding data show a similar trend in that the ligands with higher binding affinities (determined by NMR) show an increased delay in the onset of irreversible unfolding. The correlation is not perfect (e.g., ethylguanidinium appears by NMR to bind more tightly than methylguanidinium, but both cause a thermal upshift of 2.2°C). We attribute this discrepancy in part to the use of different conditions and also because the thermal unfolding was not reversible. The unfolding data are, however, also consistent with reduction in the tendency of the unfolding protein to aggregate. Stabilization of proteins against aggregation has been proposed as the mechanism by which the amino acid L-arginine facilitates the process of protein refolding (Baynes et al. 2005). Addition of a methyl and then ethyl group increasingly resembles arginine, so a nonspecific mechanism that delays aggregation may be operant as well.

The crystallographic results show that guanidinium ion and its methyl and ethyl counterparts all bind in very similar modes. All make essentially identical hydrogen bonds to the protein. Also, comparing the three ligand-bound structures among themselves, there are very

Figure 4. (A) Combined (^1H - ^{15}N) HSQC chemical shifts in L20/R63A mutant lysozyme resulting from the addition of 1 M guanidinium ion. The peak corresponding to Leu32 is highlighted. (B) Change in the ^1H - ^{15}N chemical shift of Leu32 in L20/R63A lysozyme as a function of bound guanidinium ion. The solid line shows the fit to the data for a single-site binding model. (C) Chemical shift data, as in B, for methylguanidinium. (D) Chemical shift data, as in B, for ethylguanidinium.

few changes in the conformation of the protein. Under these circumstances it would seem reasonable to assume that the changes in affinity of binding might be estimated directly from changes in the buried surface area.

Based on the X-ray structures, there is a reduction in solvent-accessible surface of each ligand, going from the free to the protein-bound form (Table 2). Comparing this

Table 1. Solubility and binding affinities for guanidinium, methylguanidinium, and ethylguanidinium ions

	Guanidinium	Methylguanidinium	Ethylguanidinium
Solubility (M)	~ 8	~ 2	~ 0.3
K_d (mM)	216 ± 28	148 ± 4	73 ± 10
ΔG_b (kcal/mol)	-0.91 ± 0.1	-1.14 ± 0.1	-1.6 ± 0.1

reduction for both alkylated guanidinium ligands and the guanidinium ion, there is a difference of $63 \text{ \AA}^2$. The extra surface being buried is due to the methyl group and is therefore hydrophobic in nature (the ethyl group of the ethylated guanidinium points away from the protein and is fully solvated; Fig. 3C). For buried hydrocarbon surfaces it is commonly accepted that each $\text{\AA}^2$ of solvent-inaccessible area generates ~ 25 cal/mol of binding energy (Creighton 1992). Using this scale, $63 \text{ \AA}^2$ corresponds to ~ 1.6 kcal/mol. The experimental increase (Table 1) is 0.7 and 0.2 kcal/mol for ethyl- and methylguanidinium. This estimate is crude at best, in part because the surface area values are small and susceptible to changes in atomic positions. Hence, the calculated energy changes are very modest and easily modulated by quite small changes in the conformation of the protein. What is encouraging,

however, is that the three ligands tested do show a progressive increase in binding affinity as the methyl and ethyl substituents are added.

Evolutionary implications

We have previously suggested that sequence duplication followed by a single mutation of the type included in mutant L20/R63A can permit alternative conformations and possibly facilitate the evolution of new function (Yousef et al. 2004). The way in which guanidinium derivatives can serve as a surrogate for an arginine side chain also suggests that substitutions of this sort might have occurred during protein evolution. A primitive and perhaps disordered protein might have been stabilized in a biologically active conformation by the binding of some adventitious ligand. Given this biologically useful variant, selective pressure would then favor amino acid substitutions that could stabilize the protein without the need for the bound ligand. These mutations might generate interactions that mimic those originally generated by the adventitious ligand.

On the rational design of a fluorescence-based biosensor

Mansoor and Farrens (2004) have characterized the use of 2-pyridyldithiobimane (PDT-Bimane) for site-directed fluorescence labeling of proteins using T4 lysozyme as a model system (Mansoor et al. 2002). The fluorescent label reacts specifically with cysteine residues via a disulfide exchange reaction.

The rationale behind the construction of the current assay was the distance-dependent quenching effect of tryptophan residues on the fluorophore. Based on the X-ray structures of the liganded and unliganded protein (Fig. 1), a tryptophan at position 39 would move closer to a fluorophore attached to residue 24 when the protein switches from the liganded to the unliganded form. This would be expected to cause a change in fluorescence, allowing the change in conformation to be monitored in solution. Thus, the triple mutant L20/R63A/I39W/Y24C was constructed with the name L20/R63A/I39W/Y24b referring to the modified protein with the label attached to Cys24. The labeled mutant L20/I39W/Y24b

Table 2. Solvent-accessible surface area of the free and bound ligands

	Solvent accessibility ($\text{\AA}^2$)		
	Guanidinium	Methylguanidinium	Ethylguanidinium
Free	132	225	256
Bound	57	88 ^a	118 ^a
Δ	75	138 ^a	138 ^a

^a Average values for the alternative conformations of the ligand.

Figure 5. Relative fluorescence emission titration curve for the mutant L20/R63A/I39W with methylguanidinium.

was also constructed and used as a control (a conformational change should not occur since the arginine at position 63 is present in the mutant).

Titration curves of the relative fluorescence intensity versus the molar concentration of the ligand were plotted (Fig. 5). The relative fluorescence intensity for the triple mutant L20/R63A/I39W/Y24b increases as a function of the ligand concentration (Fig. 5). This is consistent with the ligand triggering the conformational change and causing Trp39 to move farther from the fluorophore and diminish the quenching effect (Fig. 1). Preliminary X-ray data of the liganded switch protein with I39W mutation (L20/R63A/I39W) showed that the Trp mutation does not change the overall structure of the liganded form.

The titration data were fitted satisfactorily to a single-site model and yielded a dissociation constant of $K_d = 217 \pm 64$ mM. The affinity is somewhat different from that observed by NMR (148 ± 4 mM), presumably due to the different protein constructs used for the assays (mutant L20/R63A for NMR and L20/R63A/I39W/Y24b for fluorescence).

Materials and methods

Cloning, protein purification, and crystallization

The “switch mutant” construct L20/R63A was cloned, purified, and crystallized essentially as described previously (Yousef et al. 2004) in the presence of 200 mM of the alternate ligands (guanidinium chloride, methylguanidinium chloride, and ethylguanidinium sulfate). The liganded proteins all crystallized isomorphously with wild-type lysozyme in space group P3₂21.

X-ray data collection and model refinement

For the liganded protein crystals, cryocrystallographic data were collected at the Advanced Light Source (Beamlines 8.2.1 and 8.2.2, $\lambda \approx 1$ Å). The crystals were flash-cooled to 100 K in a nitrogen stream with 20% glycerol added to the

crystallization mother liquor as a cryoprotectant. Data were integrated and scaled by using the HKL2000 suite of programs DENZO, XDISP, and SCALEPACK (Otwinowski and Minor 1997). The structures of the liganded proteins were solved by molecular replacement (Rossmann 1972) and refined by CNS (Brünger et al. 1998). The electron density map for methylguanidinium (Fig. 3B) indicated that the ligand was binding in two alternative conformations. Because of the modest resolution of the X-ray data, no attempt was made to refine the relative occupancy. Rather, the ligand was assumed to be distributed 50:50 between the two alternative conformations and refined on this basis. Likewise, the electron density map for ethylguanidinium (Fig. 3C) also indicated two alternative binding modes, and these were refined assuming 50:50 occupancy. For both ethylguanidinium conformers the electron density for the distal methyl group is weak (Fig. 3C), indicating it to be poorly ordered. A summary of the data processing and refinement statistics is given in Table 3.

CNS (Brünger et al. 1998) was used for the solvent accessible area calculation with the default probe radius of 1.4 Å and the CNS atomic radii. PDB codes are given in Table 3.

Thermal analysis

Thermal denaturation experiments to compare the effect of 0.2 M KCl with that of 0.2 M of the ligands were done in 1.75 M Na_{0.55}K_{0.9}H_{1.55}PO₄ (pH 6.5) buffer using the methods described (Eriksson et al. 1993). Unfolding was irreversible in this buffer. Transition temperature increments were determined by direct overlay of unfolding curves at 0.015 mg/mL protein.

NMR spectroscopy

¹⁵N labeled L20/R63A mutant was purified out of inclusion bodies from cells grown in M9 minimal media with [¹⁵N]-ammonium chloride as the sole nitrogen source, and the double-labeled protein was obtained using [¹³C]-glucose and [¹⁵N]-ammonium chloride as the sole carbon and nitrogen sources

(Fischer et al. 1995). Purification was as described (Yousef et al. 2004).

Spectra of ¹³C, ¹⁵N double-labeled L20/R63A lysozyme were acquired on a Varian Inova 600 MHz NMR spectrometer. Samples contained 100 mM sodium phosphate (pH 6.7), 50 mM NaCl, 0.1 mM EDTA, 8% D₂O, 0.02% sodium azide at 25°C. Protein concentrations were 0.3 mM and 1 mM for free and guanidinium-bound (250 mM guanidinium chloride) L20/R63A, respectively. Backbone amide assignments of the unbound and guanidinium-bound protein were determined using HNCACB and CBCA(CO)NH spectra on uniformly ¹³C and ¹⁵N labeled protein. Several peaks in the unbound spectrum (most notably in the region of the helix duplication) could not be assigned on the basis of the unbound three-dimensional spectra and were assigned using a combination of the T4 wild-type assignments (Fischer et al. 1995), guanidinium-bound assignments, and an HSQC-monitored guanidine titration.

A series of HSQC spectra were acquired on 1 mM uniformly ¹⁵N labeled L20/R63A during the course of a titration with ethyl guanidine (0–285 mM), methyl guanidine (0–896 mM), or guanidine (0–1000 mM). The reduced titration range for ethylguanidinium is due to its lower solubility. However, this does not seem to affect the titration experiment since the plateau region is nearly reached by the solubility limit of the ligand.

Combined proton and nitrogen chemical shift differences for L20/R63A in the presence of the three different ligands were calculated using the formula

$$\Delta^{\text{combined}} = \left[(\Delta^1\text{H})^2 + (\Delta^{15}\text{N})^2 \right]^{1/2}$$

where $\Delta^1\text{H}$ and $\Delta^{15}\text{N}$ refer to the proton and nitrogen chemical shift values in Hz, respectively.

The measurements were taken by monitoring the two-dimensional ¹H-¹⁵N HSQC peak of residue Leu32 as a function of ligand concentration. Residue Leu32 is positioned adjacent to the duplicated helix and with corresponding peaks of good *xy* separation, with no resonance interference. The data were fitted to a single-site binding model using Kaleidagraph

Table 3. Data collection and refinement statistics for L20/R63A liganded with guanidinium (Gdm), methylguanidinium, and ethylguanidinium ions

	Gdm	methyl Gdm	ethyl Gdm
Space group	P3 ₂ 21	P3 ₂ 21	P3 ₂ 21
Unit cell dimensions (Å)	<i>a</i> = <i>b</i> = 60.71 <i>c</i> = 95.35	<i>a</i> = <i>b</i> = 60.68 <i>c</i> = 95.48	<i>a</i> = <i>b</i> = 60.73 <i>c</i> = 96.12
No. of molecules per asymmetric unit	1	1	1
Resolution range (outer shell) (Å)	30–1.45 (1.50–1.45)	30–1.7 (1.76–1.7)	30–1.8 (1.86–1.8)
No. of observations	153,690	233,015	137,016
Unique reflections	36,743	22,811	19,758
Completeness (outer shell) (%)	90 (89)	99.3 (100.0)	99.8 (99.9)
<i>R</i> _{sym} (outer shell) (%)	7.7 (22.8)	8.7 (22.2)	7.0 (36)
<i>I</i> /σ(<i>I</i>) (outer shell)	27 (3.6)	36 (8)	28 (3)
Refinement statistics			
<i>R</i> / <i>R</i> _{free} (%)	20.0/22.3	20.6/21.7	20.0/24.5
RMSD bond lengths (Å)	0.013	0.011	0.012
RMSD bond angles (°)	1.5	1.4	1.4
(φ,ψ) values, favored/disallowed (%)	93/0	91/0	93/0
PDB codes	2F2Q	2F47	2F32

(Synergy Software) and displayed using MicroCal Origin, version 6.0 (OriginLab).

Fluorescence spectroscopy

Amino acid substitutions I39W and Y24C (unique cysteine) were introduced sequentially to the protein constructs L20 and L20/R63A using a two-step QuikChange method (Stratagene). Mutagenesis primers were designed with ~20 bases flanking the mutation on both sides.

For the I39W mutation, the primers were: 5'-GGTCATT TGCTTACAAAACGTCATCATGGAATGCAGCAAAGTC C-3' and 5'-GGACTTTTGCTGCATTCCATGATGGACGTT TTGTAAGCAAATGACC-3'. For the Y24C mutation, the primers were: 5'-CTATAAAGACACAGAAGGCTGCTACA CTATTGGCATCGGTCA-3' and 5'-TGACCGATGCCAAT AGTGTAGCAGCCTTCTGTGTCTTTATAG-3'.

The mutant proteins were purified from inclusion bodies as previously described (Yousef et al. 2004).

Labeling of each mutant was carried out essentially as described by Mansoor et al. (2002) and Mansoor and Farrens (2004) using a 5–10 molar excess of the fluorescent label 2-pyridyldithiobimane (PDT-bimane) at 4°C overnight. Free label was separated from the labeled protein via gel filtration on a Pharmacia Biotech HiTrap desalting column.

Five-hundred microliters of an ~2.5 μ M solution of labeled protein were titrated with methylguanidinium chloride at 25°C using a Jobin Yvon Horiba Fluorolog in the FL3–12 TAU3 configuration. The fluorescence emission at 475 nm ($\lambda_{\text{ex}} = 392$ nm; integration time 10 sec) was observed for every titration point. The data were corrected for the dilution effect of the added ligand. The titration was repeated using the labeled “nonswitch” L20 mutant as a control. A small ligand quenching effect was observed and corrected for by subtracting the titration data of the labeled nonswitch L20 mutant from that for the “switch” mutant L20/R63A. The titration data for the mutant L20/R63A (Fig. 4) therefore reflect the ligand binding deconvoluted from quenching.

The corrected data were fitted to a single-site model using Kaleidagraph (Synergy Software) and displayed using MicroCal Origin, version 6.0 (OriginLab).

Acknowledgments

We thank Zac Wood for help with collecting the X-ray data, Sandra Greive for advice and technical assistance in collecting the fluorescence data, and F.W. (Rick) Dahlquist for access to NMR facilities. This work was supported in part by NIH grant GM21967 to B.W.M.

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