J Biomol NMR (2011) 51:83–88
DOI 10.1007/s10858-011-9543-z

ARTICLE

Siderocalin Q83 exhibits differential slow dynamics upon ligand binding

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Received: 4 May 2011 / Accepted: 23 June 2011

Abstract Siderocalin Q83 is a small soluble protein that has the ability to bind two different ligands (enterobactin and arachidonic acid) simultaneously in two distinct binding sites. Here we report that Q83 exhibits an intriguing dynamic behavior. In its free form, the protein undergoes significant micro-to-millisecond dynamics. When binding arachidonic acid, the motions of the arachidonic acid binding site are quenched while the dynamics at the enterobactin binding site increases. Reciprocally, enterobactin binding to Q83 quenches the motions at the enterobactin binding site and increases the slow dynamics at the arachidonic acid binding site. Additionally, in the enterobactin-bound state, the excited state of the arachidonic acid binding site resembles the arachidonic acid-bound state. These observations strongly suggest an allosteric regulation where binding of one ligand enhances the affinity of Q83 for the other one. Additionally, our data strengthen the emerging view of proteins as dynamic ensembles interconverting between different sub-states with distinct functionalities.

Keywords Siderocalin · Enterobactin · Arachidonic acid · Ligand binding · NMR · CPMG

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Abbreviations

NMR	Nuclear magnetic resonance
CPMG	Carr Purcell Meiboom Gill
AA	Arachidonic acid
DTT	Dithiotreitol

Introduction

The conformational dynamics of proteins is encoded in their structures and is a crucial element of their function (Karplus and Kuriyan 2005). Many protein functions and properties are intrinsically associated with protein dynamics such as folding and thermodynamic stability (Wand 2001), or exchange between conformational sub states (Kern and Zuiderweg 2003). Major contributions in the field of protein dynamics by NMR have been made by Lewis Kay and coworkers over the last two decades: from the first measurements of backbone dynamics of the staphylococcal nuclease (Kay et al. 1989) to the characterization of slow motion involved in conformational exchange (Tollinger et al. 2001) until the recent structural determination of invisible excited states (Baldwin and Kay 2009). The methodological advances introduced by Kay and coworkers allowed NMR spectroscopy to pioneer in the protein dynamics field by providing quantitative and qualitative description of protein dynamic phenomena that underlie biochemical processes. Lewis Kay particularly focused on protein dynamics, in the micro-to-millisecond timescale, that can be addressed by relaxation dispersion experiments (Palmer et al. 2001) based on the CPMG sequence (Carr and Purcell 1954; Meiboom and Gill 1958). This type of motion is often characterized by an exchange between a low energy ground state and one or more high

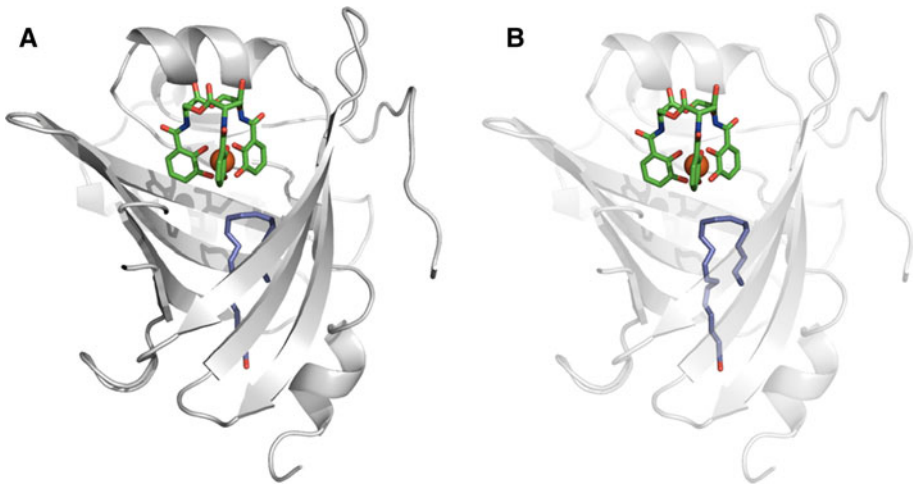

Fig. 1 Solution structure of the Q83/$[Ga^{III}(Ent)]^{3-}$/AA complex. **a** Ribbon model of the Q83/$[Ga^{III}(Ent)]^{3-}$/AA complex. The protein ribbon is shown in *grey*, enterobactin is represented as *green sticks*, Ga(III) is depicted as an *orange sphere* and AA as *blue sticks*. **b** The protein ribbon is shown in *transparency*

energy excited states. Micro-to-millisecond dynamics have been shown to be involved in many biochemical processes such as ligand binding (Mittag et al. 2003), protein folding (Tollinger et al. 2006) and allosteric regulation (Bruschweiler et al. 2009).

We have recently shown that quail lipocalin Q83 is a siderocalin (Coudevylle et al. 2010) that has the ability to bind two different ligands (enterobactin and arachidonic acid) simultaneously in two distinct binding sites. The enterobactin ligand (in our case, gallium(III)-enterobactin: $[Ga^{III}(Ent)]^{3-}$) binds to the wide-open end of the β-barrel with an affinity of 0.5 nM, whereas arachidonic acid (AA) binds to the narrow end of the β-barrel with a nanomolar affinity (Cancedda et al. 1996) (Fig. 1). We report here that the siderocalin Q83 exhibits differential slow dynamics upon ligand binding that we characterized by relaxation dispersion experiments.

Materials and methods

Sample preparation

Q83 was expressed and purified as already described (Coudevylle et al. 2010). To avoid paramagnetic effects of iron, gallium was used as a non-paramagnetic iron mimic in order to form the $[Ga^{III}(Ent)]^{3-}$ complex. The Q83/$[Ga^{III}(Ent)]^{3-}$, Q83/AA and Q83/$[Ga^{III}(Ent)]^{3-}$/AA complexes where prepared as already described (Coudevylle et al. 2010). All NMR samples were concentrated up to 0.5 mM of protein, with a slight excess of ligand (1.2 equivalent) for the bound forms, in 20 mM NaPi, 50 mM Nacl, 0.5 mM DTT, pH 6.5 supplemented with 10% D_2O.

NMR Relaxation experiments and data fitting

Backbone ^{15}N single-quantum relaxation dispersion experiments were carried out at 25°C on Varian Inova spectrometers operating at 600 and 800 MHz. CPMG-based radio frequency field strengths (ν_{CPMG}) ranged from 40 to 960 Hz with a CPMG delay of 50 ms. Duplicate data sets were recorded at selected ν_{CPMG} values for error analysis. All spectra were processed using NMRPipe/NMRDraw (Delaglio et al. 1995) and analyzed with Sparky. Relaxation data were analyzed using rdnmr (http://tollinger.spinrelax.at/) following the already described procedure (Tollinger et al. 2006; Tollinger et al. 2001).

Results and discussion

The first ^{15}N CPMG measurements made on the free form of Q83 revealed that a large majority of residues within the calyx (the canonical lipocalin ligand binding site) exhibit significant exchange contributions (R_2^{exch}), up to 5 s^{-1}, to the transverse relaxation (Fig. 2a top). $[Ga^{III}(Ent)]^{3-}$ binding to Q83 dramatically changes the micro-to-millisecond dynamics (Fig. 2a middle). Some residues exhibit lower exchange contributions while some others show a significant increase of slow dynamics (Fig 2a bottom). Surprisingly, these two different behaviors appear to topologically cluster on the structure of Q83. Residues showing a decrease of R_2^{exch} are located at the $[Ga^{III}(Ent)]^{3-}$ binding site whereas residues undergoing an increase in the exchange contribution cluster at the AA binding site (Fig. 2b). AA binding to Q83 shows the reciprocal behavior, residues from the $[Ga^{III}(Ent)]^{3-}$ binding site exhibit an increase of exchange whereas the exchange contribution for the residues in the AA binding site is nearly quenched. Finally, in the ternary complex (Q83/$[Ga^{III}(Ent)]^{3-}$/AA) micro-to-millisecond dynamics are completely quenched (data not shown). These observations clearly indicate that Q83 is undergoing micro-to-millisecond conformational exchange, giving rise to exchange contribution to the transverse relaxation rate, and that Q83

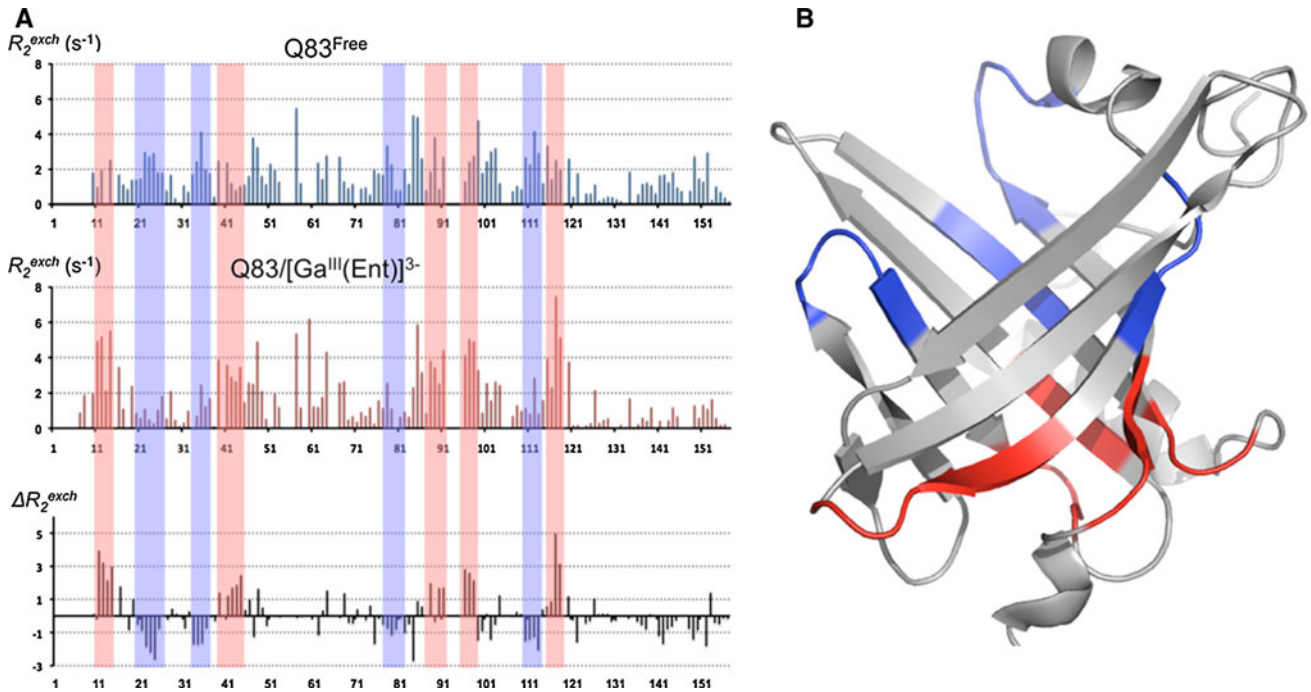

Fig. 2 Slow dynamics changes upon ligand binding. **a** Per residue exchange contribution to the transverse relaxation as a function of the protein sequence; for Q83^{Free} (*top*) and the Q83/[Ga^{III}(Ent)]$^{3-}$ complex (*middle*), the R_2^{exch} is taken as the different between R_2^{eff} at ν_{CPMG} of 40 Hz and ν_{CMPG} of 960 Hz. Difference of R_2^{exch} between the free and bound form (ΔR_2^{exch}, *bottom*), regions with an increasing dynamics are highlighted in *red* and region showing a decrease of dynamics are highlighted in *blue*. **b** Dynamic changes upon ligand binding are highlighted on the Q83 structure in *red* (increase) and *blue* (decrease)

exhibits differential conformational exchange upon ligand binding.

In order to gain further insight into the dynamic changes upon ligand binding we carried out a thorough analysis of the relaxation dispersion profiles employing the Carver formalism (Carver and Richards 1972). Consistent results were obtained assuming a two-site exchange between a ground state and an excited stat (G ↔ E). Exchange rate values (k_{ex}), populations of the excited state (P_b) and the difference in resonance frequencies between G and E states ($\Delta\omega$) were first obtained from individual residues fit. Residues at the [Ga^{III}(Ent)]$^{3-}$ binding site show clearly different k_{ex} and P_b values from residues at the AA binding site (Fig. 3). Therefore, two groups of residues were define, within which we assumed that all residues undergo the same exchange process: one group comprising residues from the [Ga^{III}(Ent)]$^{3-}$ binding site and the other group including residues from the AA binding site. A global fit was performed for these two sets of residues in the 3 different Q83 forms (Q83^{Free}, Q83/[Ga^{III}(Ent)]$^{3-}$ and Q83/AA) assuming uniform k_{ex} and P_b values but site-specific $\Delta\omega$ values. The results of the global fit are summarized in Table 1. In the free form, the [Ga^{III}(Ent)]$^{3-}$ binding site exhibits an exchange rate (k_{ex}) of 472 s^{-1} with an excited state population of 1,1%. AA binding to the free form seems to increase the exchange rate for these residues (to 1,240 s^{-1}) without significantly increasing the population of the excited state (1,1%). In the free form, the AA binding site exhibits an exchange rate of 395 s^{-1} with an excited state population of 1,4%. Upon [Ga^{III}(Ent)]$^{3-}$ binding, the AA binding site significantly changes its dynamic behavior. The exchange rate increases to 549 s^{-1} and the population of excited state increases to 2,4%. Moreover, there is a clear correlation between the experimental chemical shift changes upon AA binding and the calculated chemical shift differences between the ground and excited states of [Ga^{III}(Ent)]$^{3-}$-bound forms (Fig. 4a). This strongly suggests that in the Q83/[Ga^{III}(Ent)]$^{3-}$ complex, the excited state has a backbone conformation similar to the AA-bound form and that this state could correspond to a binding competent state. Three residues of the AA binding site do not follow this correlation, all of which are located closed to Trp$_{13}$ that undergoes a side-chain conformational change upon AA binding (Fig. 4b). Likewise, such a correlation is also not observed for the residues in the enterobactin binding site, but the aromatic nature of the ligand essentially rules out the possibility of such observation. Indeed, the chemical shift changes upon [Ga^{III}(Ent)]$^{3-}$ binding are certainly dominated by the change of chemical environment (due to the aromatic systems of enterobactin) rather than by the conformational changes of the backbone.

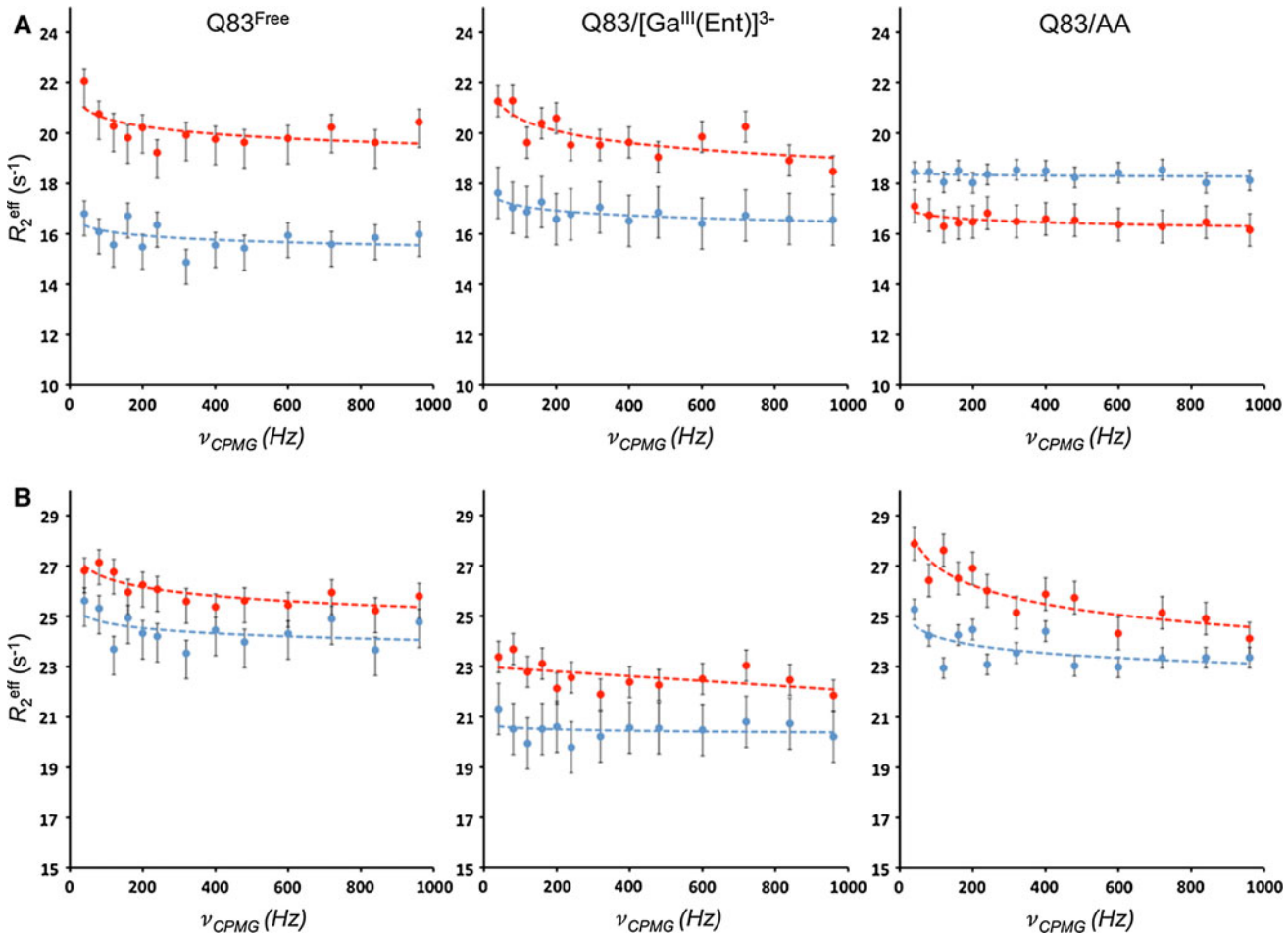

Fig. 3 Representative ^{15}N dispersion profiles for residue Arg_{72} from the AA binding site (**a**), and residue Ala_{53} from the $[Ga^{III}(Ent)]^{3-}$ binding site (**b**), recorded for the 3 different forms of Q83 at 600 and 800 MHz (*blue* and *red curves* respectively)

Table 1 Two-sites conformational exchange parameters for the two different binding sites in the three forms of Q83

		$Q83^{Free}$	$Q83/[Ga^{III}(Ent)]^{3-}$	Q83/AA	$Q83/[Ga^{III}(Ent)]^{3-}$/AA
$[Ga^{III}(Ent)]^{3-}$site	k_{ex}	472 ± 212 s^{-1}	nd	1,240 ± 342 s^{-1}	nd
	P_b	1.1 ± 0.7%	nd	1.1 ± 0.3%	nd
AA site	k_{ex}	395 ± 108 s^{-1}	549 ± 91 s^{-1}	nd	nd
	P_b	1.4 ± 0.3%	2.4 ± 0.5%	nd	nd

Conclusion

We reported here that the siderocalin Q83 exhibits an intriguing dynamic behavior. In its free form, the protein undergoes significant micro-to-millisecond dynamics. When binding AA, the motion of the AA binding site is quenched whereas the dynamics at the $[Ga^{III}(Ent)]^{3-}$ binding site increases. Reciprocally, $[Ga^{III}(Ent)]^{3-}$ binding to Q83 quenches the motions at the $[Ga^{III}(Ent)]^{3-}$ binding site and increase the slow dynamics at the AA binding site. Additionally, in the $[Ga^{III}(Ent)]^{3-}$-bound state, the excited state of the AA binding site resembles the AA-bound state.

It is becoming increasingly clear that protein dynamics and ligand binding are intimately linked. The recent methodological advances in the NMR field have allowed the comprehensive characterization of dynamic processes underlying protein/ligand interactions. Ligand binding usually leads to a decrease of flexibility at the ligand binding site with concomitant entropy loss. This entropic penalty to ligand binding can be compensated, among other possibilities, by an increased dynamics at remote sites of

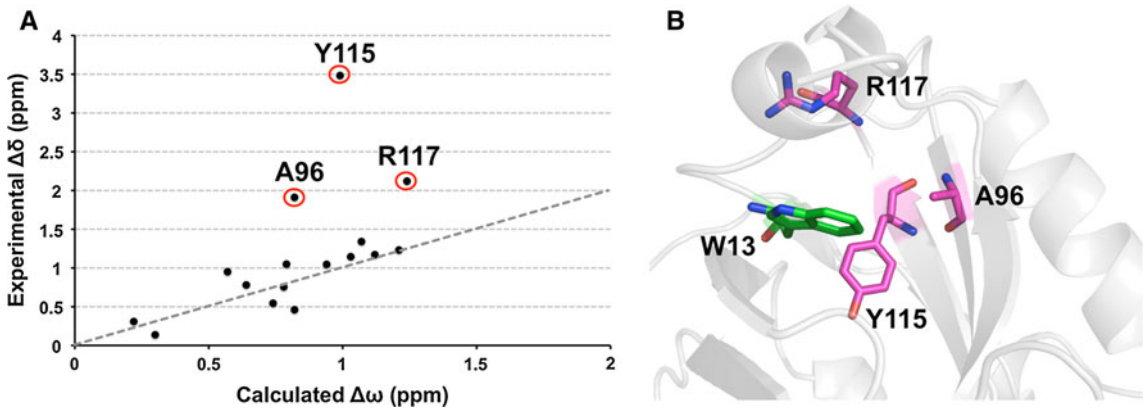

Fig. 4 The excited state of the AA binding site seems to resemble the AA-bound state. **a** Experimental chemical shift changes upon AA binding as a function of the calculated chemical shift difference between the ground and excited state of the Q83/$[Ga^{III}(Ent)]^{3-}$ complex for residues in the AA binding site. **b** Location of the three outliers on the solution structure of Q83; residues Tyr_{115}, Ala_{96} and Arg_{117} are represented as magenta sticks, residue Trp_{13} is represented as *green sticks*

the protein (MacRaild et al. 2007; Lee et al. 2000). But entropy changes upon binding are usually associated to fast dynamics changes (in the pico-to-nanosecond timescale) (Lee et al. 2000), and it is therefore unlikely that the variations in Q83 slow dynamics upon binding are related to an entropy loss compensation, especially considering that in the ternary complex all slow motions are quenched. It has been recently described that slow dynamics changes can be associated with allosteric regulation (Bruschweiler et al. 2009). In this framework, a given binding site exchanges between a highly populated grounds state and a low populated high-energy state. This high-energy state is the binding competent state. Binding of a ligand to remote site modifies the state distribution at the first site (and consequently the exchange contribution). The change of populations affects the affinity for the second ligand by increasing or decreasing the population of binding competent state. Our data strongly suggest that Q83 shows a similar behavior: binding of one ligand increases the dynamics at the other ligand binding site. This hypothesis is strongly supported by the clear correlation between experimental chemical shift changes upon AA binding and the chemical shift differences between the ground and excited states of the AA binding site in the $[Ga^{III}(Ent)]^{3-}$-bound form.

Finally, our observations add up to the growing evidence that the canonical picture of static protein structures is not an appropriate description of these entities. Indeed it is becoming obvious that proteins in solution exist as dynamic ensembles exchanging between different functional states. Only a correct description of these dynamic ensembles will lead to better understanding and description of the functional properties of proteins and other biomolecules.

Acknowledgments This work was supported by Austrian Science Fund (FWF) grants P20549-N19, P22125-B12, P17041, and P18148. NC is a recipient of a Lise Meitner FWF Fellowship. We are deeply grateful to Lewis Kay for the ingenious development and the generous distribution to the community of his pulse sequences.

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