Published in final edited form as:

Nat Struct Mol Biol. 2006 September ; 13(9): 831–838. doi:10.1038/nsmb1132.

Dynamically driven protein allostery

Nataliya Popovych¹, Shangjin Sun¹, Richard H. Ebricht², and Charalampos G. Kalodimos¹

¹Department of Chemistry, Rutgers University, Newark, New Jersey 07102

² Howard Hughes Medical Institute, Waksman Institute and Department of Chemistry, Rutgers University, Piscataway, New Jersey 08854

Abstract

Allosteric interactions are typically considered to proceed through a series of discrete changes in bonding interactions that alter the protein conformation. Here we show that allostery can be mediated exclusively by transmitted changes in protein motions. We have characterized the negatively cooperative binding of cAMP to the dimeric catabolite activator protein (CAP) at discrete conformational states. Binding of the first cAMP to one subunit of CAP dimer has no effect on the conformation of the other subunit. The dynamics of the system, however, are modulated in a distinct way by the sequential ligand binding process, with the first cAMP partially enhancing and the second cAMP completely quenching protein motions. As a result, a pronounced conformational entropic penalty is incurred by the second cAMP binding that is entirely responsible for the observed cooperativity. The results provide strong support of the existence of purely dynamics-driven allostery.

Allosteric regulation is widely used in biological systems as a very effective mechanism to control protein activity^{1–4}. Although a variety of allosteric systems have been studied for many decades, the mechanisms that underlie the communication of distant sites and energetically couple them remain largely elusive. According to the classical “mechanical” view allosteric interactions are mediated by a series of discrete conformational changes that alter protein structure^{5–7}. Allosteric regulation within or across protein subunits requires that spatially separated sites be energetically coupled. Because allostery is fundamentally thermodynamic in nature, long-range communication may be mediated not only from a change in the mean conformation (enthalpic contribution) but also from a change in the dynamic fluctuations about the mean position (entropic contribution)^{8,9}. The current view of allostery tends to explain the phenomenon only in pure structural terms, neglecting the important role of protein motions. To the other extreme, allosteric processes could, in theory, proceed solely through alteration in protein dynamics in the absence of conformational changes¹⁰. While there is increasing evidence suggesting a role of conformational dynamics in cooperative ligand binding processes^{1,11–17}, it has not been known whether dynamic phenomena can dominate allosteric mechanisms in proteins. Here, we present direct experimental evidence that allostery can be driven solely by changes in protein dynamics.

Typically, studies of systems exhibiting homotropic cooperativity have focused on comparison of unliganded beginning and ligand-bound end states. However, the key conformational state with the potential to provide detailed insight into the mechanisms that underpin the allosteric process is the intermediate state. These states cannot be isolated in positively cooperative proteins because the singly liganded intermediates are poorly populated. In contrast, negatively

*To whom correspondence should be addressed: babis@andromeda.rutgers.edu (C.G.K.).

Competing Interests Statement: The authors declare that they have no competing financial interests.

cooperative systems allow, in principle, for the isolation and characterization of intermediates states^{14,18}. We have exploited the strong negative cooperativity of cAMP binding to CAP to “freeze” binding conformations at intermediate stages and used nuclear magnetic resonance (NMR) and isothermal titration calorimetry (ITC) to assess the role of structure and dynamics in allosteric cooperativity. CAP is a transcriptional activator that exists as a homodimer in solution (K_d of the dimer is ~ 0.03 nM)¹⁹, with each subunit comprising a ligand-binding domain at the N terminus (CAP^N; Supplementary Fig. 1 online), which is also responsible for the dimerization of the protein, and a DNA-binding domain at the C terminus^{20,21}. Two cAMP molecules bind to dimeric CAP with negative cooperativity and function as allosteric effectors by increasing protein's affinity for DNA²⁰. The distance between the phosphate groups of the two bound cAMP molecules are more than 24 Å (ref. 21), so Coulombic interactions do not contribute to the negative cooperativity. cAMP likewise exhibits strong negative cooperativity in its interaction with the isolated dimeric CAP^N (ref. 22).

To directly characterize the changes in protein structure and dynamics as a function of the ligation state, we analyzed each of the three distinct conformational states of the dimeric CAP^N (residues 1-138) along the cooperative reaction coordinate: the unliganded state (apo-CAP^N), the singly liganded (intermediate) state (cAMP₁-CAP^N), and the doubly liganded state (cAMP₂-CAP^N). The isolation and characterization of such states allowed us to directly probe the effect induced by the binding of the first ligand molecule to the second, unliganded site. We found that binding of the first cAMP molecule has a minimal effect on the conformation of the second binding site. Instead, a distinct alteration of protein motions between the two sequential cAMP binding steps results in a dramatic difference in conformational entropy. Thermodynamic analysis confirms that the observed negative cooperativity is entirely driven by changes in entropy.

Results

cAMP binding to CAP^N yields distinct conformational states

We have recorded 2D ¹H-¹⁵N Heteronuclear Single Quantum Coherence (HSQC) spectra of CAP^N in three states: apo-CAP^N, cAMP₁-CAP^N, and cAMP₂-CAP^N (Fig. 1). In the apo state, CAP^N exists as a symmetric dimer with identical ligand binding sites, as evidenced by the appearance of only one set of peaks for both subunits (Fig. 1a). Binding of the first cAMP molecule to CAP^N gives rise to the intermediate state complex, cAMP₁-CAP^N; because of the strong negative cooperativity, only one of the two subunits is occupied. As expected for an asymmetric dimer, the chemical shift degeneracy is lifted and two sets of signals are observed: one set remains largely unchanged and corresponds to the unliganded subunit and the other one, markedly affected by cAMP binding, represents the liganded subunit (Fig. 1b and Supplementary Fig. 2 online). Binding of the second cAMP molecule to yield the cAMP₂-CAP^N complex, restores the symmetry of the protein dimer to a conformation identical to that of the liganded subunit in the cAMP₁-CAP^N complex (Fig. 1c). Both cAMP binding reactions are very slow on the NMR time scale.

Further analysis of the chemical shift behavior of CAP^N during its sequential interaction with cAMP (Supplementary Fig. 2) provides further unambiguous evidence of the strong negative cooperativity, in agreement with previous biochemical data²². The key to discriminating between negative and positive cooperativity is to study the complex at the stoichiometric ratio of cAMP: CAP^N (dimer) 1:1. If cAMP bound to CAP^N with positive cooperativity, two species would be present at this ratio; the apo-CAP^N and the cAMP₂-CAP^N. Since both cAMP binding steps are slow on the NMR time scale, 50% of the CAP^N would remain in the apo state, that is, the resonances would not be affected by the presence of the cAMP. However, this is not what we observe experimentally. The unfilled subunit at the ratio of cAMP: CAP^N 1:1 is clearly affected, as many residues at the interface with the liganded subunit change substantially their

chemical shift (Supplementary Fig. 2). These results demonstrate that there is only one protein complex present in solution at the cAMP: CAP^N 1:1 ratio corresponding to the intermediate cAMP₁-CAP^N complex. Therefore, cAMP binds to CAP with strong negative cooperativity.

Effect of sequential cAMP binding on the structure of CAP^N

To assess the effect on protein structure of the sequential binding of the first and second cAMP molecules to CAP^N, we have used chemical shift mapping. The exquisite sensitivity of the chemical shift to environment ensures that considerable shifts would take place if changes in the mean protein conformation occurred on ligand binding. The effect of first cAMP binding to the liganded subunit is pronounced throughout the whole monomer as evidenced by the observed widespread chemical shift changes (Fig. 2). In contrast, the unliganded subunit in the cAMP₁-CAP^N complex is slightly perturbed only at its long C helix, which forms the dimer interface. None of the residues in the β -roll or the phosphate binding cassette (PBC) motif, the most conserved and important region in the protein for its interaction with cAMP, is affected. Further examination of the chemical shift behavior of CAP^N during its sequential interaction with cAMP demonstrates that the effect of cAMP binding on protein structure is confined, in each step, only to the interacting subunit (Supplementary Fig. 2). Thus, the mean conformation of the unfilled cAMP site at the unliganded subunit of the cAMP₁-CAP^N complex is not at all affected by the presence of the first cAMP, suggesting that the contribution of the “structural” component to the observed negative cooperativity is negligible.

Effect of sequential cAMP binding on slow protein motions

To understand how protein dynamics adjust along the cooperative reaction coordinate, we characterized the backbone motions of CAP^N as a function of the cAMP ligation state over a wide range of functionally relevant time scales by measuring relaxation rates (Supplementary Fig. 3 online). Slow domain motions on the micro- to millisecond (μ s-ms) time regime are biologically very important because they are close to the time scales on which functional processes take place, such as docking, protein folding and allosteric transitions²³⁻²⁶. Upon binding of the first cAMP to CAP^N, resonances for backbone residues of both subunits undergo extensive peak broadening (Supplementary Fig. 4 online). This indicates the presence of substantial conformational exchange, which is identified by the high magnitude of the term R_{ex} (Fig. 3). The contribution to R_{ex} from cAMP binding and dissociation is negligible (see Supplementary Discussion). Thus, the pronounced increase of R_{ex} for almost all residues of both subunits means that the protein in the cAMP₁-CAP^N complex populates an ensemble of alternate conformations that interconvert on the μ s-ms timescale. These slow motions are activated by the first cAMP binding.

Notably, despite the fact that binding of the first cAMP molecule cannot induce long-range structural effects to the other subunit (Fig. 2), the dynamics of the system respond to the binding in a highly cooperative way emphasizing their allosteric character. Residues of the unliganded subunit in the cAMP₁-CAP^N complex that are located as far as 35 Å from the bound cAMP exhibit enhanced dynamics (e.g., Tyr23, Leu29, Ile30). Strikingly, the prominent conformational dynamics on the μ s-ms time scale exhibited by almost all residues in cAMP₁-CAP^N are completely quenched upon binding of the second cAMP molecule to yield the cAMP₂-CAP^N complex (Fig. 3).

Both subunits undergo the same conformational exchange

To better understand the conformational process that results in such a dramatic change in slow motions of the singly liganded cAMP₁-CAP^N complex, we performed a series of relaxation dispersion experiments (Fig. 4)²⁷. The additional line-broadening of NMR signals (R_{ex}) caused by conformational exchange between the ground and an excited state depends on the sum of forward and reverse rates of interconversion (k_{ex}), the relative populations of the exchanging

species (p_A and p_B) and the chemical shifts between the exchanging species ($\Delta\omega$)²⁷. Because the conformational exchange process is in the fast exchange limit, only k_{ex} and the product $p_{APB}(\Delta\omega)^2$ can be measured. The results show that k_{ex} appears to be similar for most of the residues in the liganded subunit as the majority of the residues with appreciable R_{ex} can be fitted with k_{ex} values ranging from 900 to 1600 s⁻¹ (Fig. 4a). Therefore, it appears that most of the residues in the liganded subunit report on the same exchange process. Assuming a population of 10% ($p_B \sim 0.1$) for the excited state, an estimation of $\Delta\omega$ can then be made. The ¹⁵N chemical shift difference ($\Delta\omega$) between the two exchanging conformations may be as high as 4 ppm for residues with large R_{ex} values (~ 10 -15 Hz), implying major structural rearrangements. If the population of the excited state (p_B) is lower than 10%, then even larger $\Delta\omega$ values would be present. As in the case of the liganded subunit, k_{ex} for most residues in the unliganded subunit can be fitted using k_{ex} values ranging from 1000 to 1500 s⁻¹. Another smaller group of residues in the unliganded subunit (e.g. Ile106; Fig. 4b) appear to have k_{ex} values close to 2,000 s⁻¹. It is possible that these few residues are affected by another, faster process. However, considering the uncertainty, almost all residues can be considered to exchange between the sites with similar k_{ex} . Therefore, it is very likely that all residues of the cAMP₁-CAP^N complex, both from the liganded and the unliganded subunits, report on the same conformational exchange process. This is corroborated by the observation that residues located at the dimer interface have very similar k_{ex} .

Effect of sequential cAMP binding on fast protein motions

Next, we measured changes in the pico- to nanosecond (ps-ns) timescale motions of the backbone of CAP^N as a function of the ligation state (Fig. 5). Motions on this fast time regime are biologically very important because of their strong effect on the entropy of the system^{28, 29}. Even small changes of these motions have been shown to result in significant entropic changes capable of driving binding processes³⁰⁻³². The conformational flexibility of CAP^N on the ps-ns time scale, as indicated by the spectral density function $J(0.87\omega_H)$, is illustrated in Figure 5. Our data show that binding of the first cAMP molecule to yield the cAMP₁-CAP^N complex does not affect these motions appreciably (Fig. 5). The effect is local and is confined within the cAMP binding site, where only few residues rigidify their backbone. Residues 80-84 at the PBC motif of the liganded subunit and 126-133 at the C terminus of the unliganded subunit are the only ones that slightly dampen their fast motions in the singly liganded complex. In contrast to the slow motions (Fig. 3), the ps-ns dynamics of the core of the unliganded subunit are not perturbed. Notably, binding of the second cAMP molecule causes widespread stiffening of the backbone fluctuations in both subunits (Fig. 5). The extensive suppression of protein motions throughout the whole molecule attests, once again, to the allosteric nature of protein motions and their exceptional capacity to serve as a carrier of allosteric energy

Effect of cAMP binding on the native-state ensemble

To directly probe the native-state conformational ensemble of CAP^N, we measured NMR-detected hydrogen/deuterium (H/D) exchange rates of the backbone amides of CAP^N as a function of the cAMP ligation state. The native-state ensemble of a protein consists of an equilibrium of conformational states and its redistribution upon ligand binding has been suggested to impinge on cooperativity^{11,12,33-35}. Fluctuations of the structures allow the amide sites to sample a manifold of conformations, some of which permit exchange with the solvent. Therefore, amide exchange rates can reflect dynamic motions over a wide range of time scales that are not accessible by relaxation methods. Binding of the first cAMP molecule to CAP^N results in a slight decrease in exchange rates of the residues in the liganded subunit (Fig. 6). However, the unliganded subunit remains largely unaffected. Strikingly, binding of the second cAMP molecule results in substantial and global decrease in exchange rates of residues in both subunits. Residues at the dimer interface show the most pronounced increase in protection from solvent, indicating optimization of the packing interactions at the interface only when both

subunits are filled with cAMP. We conclude that a considerable reduction in the number of conformational states occurs in the cAMP₂-CAP^N complex, but not in the intermediate cAMP₁-CAP^N complex. It is again noteworthy the distinct effect that the sequential cAMP binding has on the stability of the protein, with only the second cAMP being capable of eliciting long-range effects.

Thermodynamic basis of the negatively cooperative binding

To determine the thermodynamic basis for the observed anti-cooperative binding of cAMP to CAP^N, we used high-sensitivity ITC to measure the association free energy ΔG and its enthalpic and entropic components, ΔH and ΔS . The biphasic nature of the isotherm (Fig. 7) clearly points to the existence of two non-equivalent sites in CAP^N with a large difference in the affinity for cAMP. The difference in the free energy change between the two binding steps, $\Delta G_1 - \Delta G_2$, is $-2.8 \text{ kcal mol}^{-1}$ resulting in the second cAMP molecule binding to CAP^N with a 100-fold lower affinity than the first cAMP. The second cAMP binding is accompanied by a more favorable enthalpy change, which, in line with the chemical shift mapping results, argues against its site being distorted as a result of the first cAMP binding. In fact, the measured enthalpy change between the two binding steps ($\Delta H_1 - \Delta H_2 = +1.1 \text{ kcal mol}^{-1}$) opposes the observed allosteric phenomenology and favors a positive, rather than a negative cooperativity. Instead, the second cAMP binds to CAP^N with a drastically less favorable entropy change than the first cAMP, amounting to an entropic penalty of $\Delta(-T\Delta S) = 4 \text{ kcal mol}^{-1}$. Thus, the thermodynamic basis for the observed negative cooperativity is entirely of entropic nature.

Conformational entropy change upon cAMP binding

The present findings suggest that the observed extensive changes in protein motions is the most probable source of the large difference in entropy change between the two cAMP binding steps. To assess the contribution of protein motions to the conformational entropy of the system^{36, 37}, we calculated changes in order parameters for the first and second cAMP binding to CAP^N (Fig. 8 and Supplementary Figure 5 online). The order parameter, S^2 , is a measure of the amplitude of the internal motions on the ps-ns time scale and may vary from $S^2=1$, for a bond vector having no internal motion, to $S^2=0$, for a bond vector rapidly sampling multiple orientations²⁸. Binding of the first cAMP to CAP^N results in small increases in S^2 , confined primarily to the binding site (Fig. 8). In contrast, binding of the second cAMP molecule causes a widespread increase in S^2 (Fig. 8) indicating a global rigidification of the protein, in agreement with the $J(0.87\omega_H)$ data (Fig. 5). By converting order parameters to entropy (see Methods) we estimate that binding of the first cAMP molecule to CAP^N is accompanied by a $-T\Delta S_{\text{conf}1} = 3.2 \text{ kcal mol}^{-1}$ conformational entropic penalty, whereas binding of the second cAMP molecule incurs a much higher entropic penalty, $-T\Delta S_{\text{conf}2}$, amounting to $18.1 \text{ kcal mol}^{-1}$ (Fig. 8). We should note here that conformational entropy estimated through order parameters should be taken as an upper-bound estimate, because the approach assumes that motions are uncorrelated^{28,32}. Thus, the extensive difference in S^2 is translated to an enormous decrease in conformational entropy of the system, amounting to $-T\Delta S_{\text{conf}} \sim 15 \text{ kcal mol}^{-1}$, in going from the cAMP₁-CAP^N to the cAMP₂-CAP^N state. We conclude that the calorimetrically measured pronounced entropic penalty that accompanies the anti-cooperative cAMP binding is indeed dominated by the unfavorable conformational entropy change incurred by the second cAMP binding to CAP^N.

Discussion

It is generally thought that changes in protein shape and bonding interactions, which are considered to primarily contribute to the enthalpy of the system, are necessary to propagate the binding signal to remote sites. Nevertheless, it has long been recognized the possibility of allosteric regulation through dynamic (entropic) mechanisms^{8,10,38}. Based on the present data

(summarized in a model depicted in Figure 9) we suggest that the cooperative binding of cAMP to CAP^N provides the first definitive example of a biological system wherein allosteric interactions are exclusively mediated by changes in protein motions.

Study of biological systems exhibiting negative cooperativity allows, in principle, for the isolation and characterization of intermediate states. Although limited, crystallographic studies of such oligomeric systems have indicated that binding of the first ligand may distort the shape of the unliganded subunit, thereby rendering the binding of the second ligand more unfavorable^{18,39}. Nevertheless, other crystallographic studies have failed to reveal any obvious structural effect on the unliganded subunit in the intermediate complex that could account for the observed negative cooperativity^{18,40,41}, raising the possibility that other mechanisms may act to increase the energy of the second binding step. Our NMR data show that indeed negative cooperativity can proceed with no conformational changes transmitted between the binding sites of the subunits.

Despite the fact that binding of cAMP to CAP^N cannot induce long-range structural effects to the other subunit (Fig. 2), the motions of residues located at remote sites are clearly affected (Figs. 3, 5). This observation is very important because based solely on structural information provided by the perturbation of the chemical shift, which is a very sensitive probe of changes in the average conformation, someone would erroneously conclude that the core of the unliganded subunit does not “sense” the presence of the ligand in the other subunit. It is particularly remarkable that although binding of the second cAMP molecule does not perturb the chemical shift of the other subunit, the motions of almost all residues throughout both subunits are extensively suppressed. For example, despite the fact that the unliganded subunit does not alter its mean conformation upon binding of the first cAMP (because $\Delta\delta \sim 0$), it exhibits enhanced flexibility involving excursions into alternate conformational states.

Very interestingly, slow and fast motions of residues located at distant regions are affected in the absence of a visible connectivity pathway. This result further undermines the mechanical view of allosteric cooperativity, wherein binding effects are assumed to propagate through a series of conformational distortions. As it becomes apparent from Figs. 3 and 5, the ligand-induced redistribution of the dynamic fluctuations of the protein affects regions linked by cooperative interactions, thereby providing a means of propagating the allosteric signal at the distal site even in the absence of structural changes.

The thermodynamic basis of the observed anti-cooperative binding of cAMP to CAP^N is entirely of entropic nature (Figs. 7, 9). Estimation of the conformational entropy through changes of order parameters (Fig. 8) indeed confirmed that the calorimetrically measured difference in entropy between the two sequential binding steps is primarily due to alterations in protein flexibility. Despite the large unfavorable conformational entropy resulting from the suppression of protein motions, binding of the second cAMP is still overall entropy driven, as is the binding of the first cAMP molecule, most likely because of the large favorable solvation entropy that accompanies the binding of hydrophobic ligands⁹. However, the larger conformational entropic penalty in the second step decreases drastically the total entropy of the system resulting in weaker, and, thus, anti-cooperative binding.

Earlier NMR studies have offered valuable insight into the potential role of dynamics in the function of two cooperative systems. Maler *et al.* demonstrated that calcium binding to the singly calcium-liganded complex of the dimeric calbindin D_{9k} results in suppression of fast motions in both subunits¹⁵. Despite the fact that considerable structural changes accompanied binding of calcium to calbindin D_{9k}, and that the thermodynamic basis of the cooperative binding remains unknown, the results highlight the potentially important role of protein motions. In another elegant study, Stevens *et al.* characterized the anti-cooperative ligand

binding to a dimeric enzyme¹⁴. Although the limited stability of the system didn't permit the authors to measure fast motions and order parameters, they demonstrated that slow (μ s-ms) time-scale motions are quenched only upon binding of the second ligand¹⁴. Regrettably, there is currently no theory to relate these motions to the spatial amplitude of the underlying conformational phenomena, and therefore no quantitative assessment of entropy can be made. As has been noted⁸, μ s-ms dynamics are typically considered to reflect cooperative transitions between two states, implying that the contribution of these dynamic modes to the change in entropy may be very small.

Overall, the cooperative binding of cAMP to CAP^N provides the first definitive example of a system wherein allosteric interactions are exclusively mediated by changes in protein motions. Therefore, the present findings provide direct support of the existence of biological systems that exhibit purely dynamics-driven allostery. The large amount of residual entropy detected experimentally in proteins^{8,9,42} suggests that such an allosteric mechanism may be more common than we thought.

Methods

Sample preparation

The His₆-tagged 1-138 domain (CAP^N) was amplified with the polymerase chain reaction (PCR), using as a template the pAKCRP plasmid that encodes for wild-type *E. coli* CAP (residues 1-209), and transformed in BL21(DE3) cells. Isotopically labelled samples for NMR studies were prepared by growing the cells in M9 media in the presence of ampicillin. For the backbone assignment a U-²H/¹⁵N/¹³C-labeled sample was prepared by supplementing the growing media with 1 g l⁻¹ of ¹⁵NH₄Cl, 2 g l⁻¹ ²H7/¹³C₆-glucose in 99.9%-²H₂O. For the relaxation experiments a U-²H/¹⁵N-labeled sample was prepared using 1 g l⁻¹ of ¹⁵NH₄Cl, 2 g l⁻¹ ²H7/¹²C₆-glucose in 99.9%-²H₂O. Two purification steps were used for all protein samples. The first one involved a nickel-chelated sepharose fast flow resin (Amersham) and the second one a Superdex-200 size exclusion column (Amersham). Protein concentration was determined spectrophotometrically at 278 nm using an extinction coefficient of 40,800 M⁻¹ cm⁻¹. cAMP concentration was determined spectrophotometrically using a molecular extinction coefficient of 14,650 M⁻¹ cm⁻¹ at 259 nm. Protein concentration for samples used for NMR studies was typically 0.4 mM. The singly (cAMP1-CAP^N) and doubly (cAMP₂-CAP^N) liganded complexes were prepared by using cAMP: CAP^N molar ratios of 1:1 and 2:1, respectively. The strong binding of cAMP to CAP^N for both steps (K_{d1} =0.04 μ M and K_{d2} =4 μ M, for the first and second binding steps, respectively) and the strong negative cooperativity ensures that at cAMP: CAP^N ratio of 1:1 one subunit of CAP^N is completely saturated and the other one completely empty. Similarly, at cAMP: CAP^N ratio of 2:1 both subunits are saturated, in the absence of excess of cAMP.

NMR assignment

All NMR spectra were recorded at 30 °C on a Varian 600 MHz spectrometer. Sequential assignment of the ¹H, ¹³C, and ¹⁵N protein chemical shifts in the apo, (cAMP)₁-CAP^N, and (cAMP)₂-CAP^N was achieved by means of through-bond heteronuclear scalar correlations along the backbone using a set of 3D HNCA, HN(CO)CA, HNCACB, and HN(CO)CACB experiments.

Relaxation measurement and analysis

Four relaxation parameters were measured for all backbone amides of CAP^N in the apo, singly liganded and doubly liganded complexes with cAMP: the {¹H}-¹⁵N nuclear Overhauser effect (NOE), the longitudinal relaxation time T₁, the transverse relaxation time T₂, and the rotating-frame longitudinal relaxation time T₁ ρ . ¹⁵N relaxation dispersion T₂-CPMG data were recorded

for the cAMP₁-CAP^N complex. Data sets were acquired as 256×1024 complex points in the $t_1 \times t_2$ dimensions. Data points were fitted as a function of the length of the parametric relaxation delay to two-parameter decay curves of the form $I(t) = I_0 \exp(-Rt)$. The error in the rate constant was assessed from Monte Carlo simulations. $\{^1\text{H}\}$ - ^{15}N NOE data were obtained by recording, in an interleaved manner, one spectrum with a 5 s recycle delay followed by 5 s saturation and another spectrum with no saturation and a 10 s recycle delay. Representative relaxation data were recorded at three different protein concentrations (0.05, 0.4, and 1.05 mM) to exclude contributions from protein aggregation. The final relaxation data were recorded using a protein sample of 0.4 mM concentration.

To characterize and quantify exchange contributions, R_{ex} , both the rotating frame⁴⁴ and T_2 -CPMG²⁷ relaxation dispersion experiments were recorded. In both cases R_{ex} is measured independently of the presence of anisotropy and any change that ligand binding may elicit to the overall tumbling of the protein. These NMR experiments measure the excess transverse relaxation rate, R_{ex} , caused by the exchange of nuclei between different conformations/states with different chemical shifts. The similarity of exchange rates extracted from fits of dispersion profiles on a per-residue basis (Fig. 4) supports a two-state model of exchange.

To probe fast motions that take place on ps-ns time-scale we have used the reduced spectral density mapping approach⁴⁵, which makes no assumptions a priori regarding the molecular anisotropy or internal motions. Briefly, the relation between the relaxation rates and the spectral density function, $J(\omega)$, is used to extract values of the latter with the use of T_1 , T_2 , and cross-relaxation rate, σ . At high frequencies (>400 MHz), $J(\omega)$ varies very little and the three spectra densities $J(\omega_{\text{H}} + \omega_{\text{N}})$, $J(\omega_{\text{H}})$, $J(\omega_{\text{H}} - \omega_{\text{N}})$ may be averaged to one average spectra density $\langle J(\omega_{\text{H}}) \rangle$, in which ω_{H} and ω_{N} is the ^1H and ^{15}N Larmor frequency, respectively. The frequency in $\langle J(\omega_{\text{H}}) \rangle$ may be taken equal to $0.87\omega_{\text{H}}$. The value of the spectral density function at $0.87\omega_{\text{H}}$ frequency is the most diagnostic for ps-ns motions.

Assessment of conformational entropy ($-T\Delta S_{\text{conf}}$) from order parameters

The contribution to the conformational entropy of protein motions to the cooperative binding of cAMP to CAP^N was estimated by measuring changes in the order parameter, S^2 , as a function of the ligation state. Because of the complex dynamics observed in the CAP system, reduced-spectral density mapping was employed for calculating S^2 , as described before^{46,47}. This approach was successfully applied before in calculating the conformational entropy contribution to the association entropy of a protein-DNA complex⁴⁷. A local overall rotational correlation time, τ_{M} , is obtained from the spectral density function as:

$$\tau_{\text{M}} = \omega_{\text{N}}^{-1} \left(\frac{J(0) - J(\omega_{\text{N}})}{J(\omega_{\text{N}})} \right)^{1/2} \quad (1)$$

Once a value of τ_{M} has been determined, the amplitude of intramolecular motion of the N-H bond vector in a molecular reference frame is given by:

$$S^2 = \frac{5(J(0) - J(\omega_{\text{N}}))(1 + \omega_{\text{N}}^2 \tau_{\text{M}}^2)}{2\omega_{\text{N}}^2 \tau_{\text{M}}^3} \quad (2)$$

As shown previously, the parameter of S^2 of equation (2) is a very good approximation of the square of the generalized order parameter extracted by the model-free spectral density function⁴⁶⁻⁴⁸. In the case of ribonuclease H, for example, S^2 values calculated from Eq. 2 and

using the full model-free formalism have a linear correlation coefficient of 0.97 (ref. 47). The conformational entropy of the CAP^N backbone is then calculated by³⁶:

$$\Delta S = -k_B \sum_i \ln \left\{ \frac{3 - (1 + 8S_{a,i})^{1/2}}{3 - (1 + 8S_{b,i})^{1/2}} \right\} \quad (3)$$

in which k_B is Boltzmann's constant, and S_a and S_b are the order parameters for state a (e.g. apo-CAP^N) and state b (e.g., cAMP₁-CAP^N). Accurate extraction of order parameters is critically dependent on identifying and separating the chemical-exchange contribution. In our study, this has been done by recording rotating frame and T₂-CPMG relaxation dispersion, so the T₂ values used for obtaining the $J(0)$ have been corrected for the exchange term R_{ex} .

Determination of exchange rates

Amide proton exchange rates were determined from the time course of the peak intensities in a series of ¹H-¹⁵N HSQC spectra after dissolving lyophilized samples in D₂O. The final pD was adjusted by DCl or NaOD solutions and was measured with a glass electrode at the temperature of the exchange experiment, taking into account the isotope effect: pD = pH_{read} + 0.4. Exchange rates were determined by plotting the intensities of each residue against time and fit by a first-order rate expression.

ITC experiments

Calorimetric titrations of CAP^N with cAMP were performed on a VP-ITC microcalorimeter (MicroCal). Protein samples were extensively dialyzed against the ITC buffer containing 50 mM KPi (pH 6.0), 500 mM KCl, and 1 mM TCEP. All solutions were filtered using membrane filters (pore size 0.45 μm) and thoroughly degassed for 20 min by gentle stirring under vacuum. The 1.35 sample cell was filled with a 30 μM solution of protein and the 250-μl injection syringe with 0.6 mM of the titrating cAMP. The ligand solution was prepared by dissolving cAMP in the flow through of the last buffer exchange. Each titration typically consisted of a preliminary 2-μl injection followed by 30 subsequent 8-μl injections. Data for the preliminary injection, which are affected by diffusion of the solution from and into the injection syringe during the initial equilibration period, were discarded. Binding isotherms were generated by plotting heats of reaction normalized by the moles of injectant versus the ratio of total injectant to total protein per injection. The data were fitted using the sequential binding site model embedded in Origin 7.0 (Microcal).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We wish to thank P. Loria and P. Huskey for providing us with scripts for the analysis of some of the relaxation data. This work was supported by US National Science Foundation grant MCB-0618259 to C.G.K.

References

1. Hardy JA, Wells JA. Searching for new allosteric sites in enzymes. *Curr Opin Struct Biol* 2004;14:706–715. [PubMed: 15582395]
2. Gao ZG, Jacobson KA. Allostery in membrane receptors. *Drug Discov Today* 2006;11:191–202. [PubMed: 16580596]

3. Swain JF, Gierasch LM. The changing landscape of protein allostery. *Curr Opin Struct Biol* 2006;16:102–108. [PubMed: 16423525]
4. Suel GM, Lockless SW, Wall MA, Ranganathan R. Evolutionarily conserved networks of residues mediate allosteric communication in proteins. *Nat Struct Biol* 2003;10:59–69. [PubMed: 12483203]
5. Koshland DE Jr. Conformational changes: how small is big enough. *Nat Med* 1998;4:1112–1114. [PubMed: 9771734]
6. Changeux JP, Edelstein SJ. Allosteric mechanisms of signal transduction. *Science* 2005;308:1424–1428. [PubMed: 15933191]
7. Bray D, Duke T. Conformational spread: the propagation of allosteric states in large multiprotein complexes. *Annu Rev Biophys Biomol Struct* 2004;33:53–73. [PubMed: 15139804]
8. Wand AJ. Dynamic activation of protein function: a view emerging from NMR spectroscopy. *Nat Struct Biol* 2001;8:926–931. [PubMed: 11685236]
9. Homans SW. Probing the binding entropy of ligand-protein interactions by NMR. *Chembiochem* 2005;6:1585–1591. [PubMed: 16038002]
10. Cooper A, Dryden DT. Allostery without conformational change. A plausible model *Eur Biophys J* 1984;11:103–109.
11. Freire E. The propagation of binding interactions to remote sites in proteins: analysis of the binding of the monoclonal antibody D1.3 to lysozyme. *Proc Natl Acad Sci USA* 1999;96:10118–10122. [PubMed: 10468572]
12. Pan H, Lee JC, Hilser VJ. Binding sites in *Escherichia coli* dihydrofolate reductase communicate by modulating the conformational ensemble. *Proc Natl Acad Sci USA* 2000;97:12020–12025. [PubMed: 11035796]
13. Kern D, Zuiderweg ER. The role of dynamics in allosteric regulation. *Curr Opin Struct Biol* 2003;13:748–757. [PubMed: 14675554]
14. Stevens SY, Sanker S, Kent C, Zuiderweg ER. Delineation of the allosteric mechanism of a cytidyltransferase exhibiting negative cooperativity. *Nat Struct Biol* 2001;8:947–952. [PubMed: 11685240]
15. Maler L, Blankenship J, Rance M, Chazin WJ. Site-site communication in the EF-hand Ca^{2+} -binding protein calbindin D9k. *Nat Struct Biol* 2000;7:245–250. [PubMed: 10700285]
16. Lee AL, Kinnear SA, Wand AJ. Redistribution and loss of side chain entropy upon formation of a calmodulin-peptide complex. *Nat Struct Biol* 2000;7:72–77. [PubMed: 10625431]
17. Fuentes EJ, Der CJ, Lee AL. Ligand-dependent dynamics and intramolecular signaling in a PDZ domain. *J Mol Biol* 2004;335:1105–1115. [PubMed: 14698303]
18. Koshland DE Jr. The structural basis of negative cooperativity: receptors and enzymes. *Curr Opin Struct Biol* 1996;6:757–761. [PubMed: 8994875]
19. Brown AM, Crothers DM. Modulation of the stability of a gene-regulatory protein dimer by DNA and cAMP. *Proc Natl Acad Sci USA* 1989;86:7387–7391. [PubMed: 2552443]
20. Harman JG. Allosteric regulation of the cAMP receptor protein. *Biochim Biophys Acta* 2001;1547:1–17. [PubMed: 11343786]
21. Passner JM, Schultz SC, Steitz TA. Modeling the cAMP-induced allosteric transition using the crystal structure of CAP-cAMP at 2.1 Å resolution. *J Mol Biol* 2000;304:847–859. [PubMed: 11124031]
22. Heyduk E, Heyduk T, Lee JC. Intersubunit communications in *Escherichia coli* cyclic AMP receptor protein: studies of the ligand binding domain. *Biochemistry* 1992;31:3682–3688. [PubMed: 1314647]
23. Akke M. NMR methods for characterizing microsecond to millisecond dynamics in recognition and catalysis. *Curr Opin Struct Biol* 2002;12:642–647. [PubMed: 12464317]
24. Volkman BF, Lipson D, Wemmer DE, Kern D. Two-state allosteric behavior in a single-domain signaling protein. *Science* 2001;291:2429–2433. [PubMed: 11264542]
25. Kalodimos CG, et al. Structure and flexibility adaptation in nonspecific and specific protein-DNA complexes. *Science* 2004;305:386–389. [PubMed: 15256668]
26. Keramisanou D, et al. Disorder-order folding transitions underlie catalysis in the helicase motor of SecA. *Nat Struct Mol Biol* 2006;13:594–602. [PubMed: 16783375]

27. Mulder FA, Mittermaier A, Hon B, Dahlquist FW, Kay LE. Studying excited states of proteins by NMR spectroscopy. *Nat Struct Biol* 2001;8:932–935. [PubMed: 11685237]
28. Forman-Kay JD. The ‘dynamics’ in the thermodynamics of binding. *Nat Struct Biol* 1999;6:1086–1087. [PubMed: 10581541]
29. Cavanagh J, Akke M. May the driving force be with you--whatever it is. *Nat Struct Biol* 2000;7:11–13. [PubMed: 10625416]
30. Zidek L, Novotny MV, Stone MJ. Increased protein backbone conformational entropy upon hydrophobic ligand binding. *Nat Struct Biol* 1999;6:1118–1121. [PubMed: 10581552]
31. Stone MJ. NMR relaxation studies of the role of conformational entropy in protein stability and ligand binding. *Acc Chem Res* 2001;34:379–388. [PubMed: 11352716]
32. Igumenova TI, Frederick KK, Wand AJ. Characterization of the fast dynamics of protein amino Acid side chains using NMR relaxation in solution. *Chem Rev* 2006;106:1672–1699. [PubMed: 16683749]
33. Hilser VJ, Garcia-Moreno EB, Oas TG, Kapp G, Whitten ST. A Statistical Thermodynamic Model of the Protein Ensemble. *Chem Rev* 2006;106:1545–1558. [PubMed: 16683744]
34. Gekko K, Obu N, Li J, Lee JC. A linear correlation between the energetics of allosteric communication and protein flexibility in the Escherichia coli cyclic AMP receptor protein revealed by mutation-induced changes in compressibility and amide hydrogen-deuterium exchange. *Biochemistry* 2004;43:3844–3852. [PubMed: 15049691]
35. Englander JJ, Louie G, McKinnie RE, Englander SW. Energetic components of the allosteric machinery in hemoglobin measured by hydrogen exchange. *J Mol Biol* 1998;284:1695–1706. [PubMed: 9878380]
36. Yang D, Kay LE. Contributions to conformational entropy arising from bond vector fluctuations measured from NMR-derived order parameters: application to protein folding. *J Mol Biol* 1996;263:369–382. [PubMed: 8913313]
37. Bracken C, Carr PA, Cavanagh J, Palmer AG 3rd. Temperature dependence of intramolecular dynamics of the basic leucine zipper of GCN4: implications for the entropy of association with DNA. *J Mol Biol* 1999;285:2133–2146. [PubMed: 9925790]
38. Gunasekaran K, Ma B, Nussinov R. Is allostery an intrinsic property of all dynamic proteins? *Proteins* 2004;57:433–443. [PubMed: 15382234]
39. Anderson AC, O’Neil RH, DeLano WL, Stroud RM. The structural mechanism for half-the-sites reactivity in an enzyme, thymidylate synthase, involves a relay of changes between subunits. *Biochemistry* 1999;38:13829–13836. [PubMed: 10529228]
40. Leslie AG, Wonacott AJ. Structural evidence for ligand-induced sequential conformational changes in glyceraldehyde 3-phosphate dehydrogenase. *J Mol Biol* 1984;178:743–772. [PubMed: 6492162]
41. Hampele IC, et al. Structure and function of the dihydropteroate synthase from Staphylococcus aureus. *J Mol Biol* 1997;268:21–30. [PubMed: 9149138]
42. Lee AL, Wand AJ. Microscopic origins of entropy, heat capacity and the glass transition in proteins. *Nature* 2001;411:501–504. [PubMed: 11373686]
43. Evenas J, et al. Ligand-induced structural changes to maltodextrin-binding protein as studied by solution NMR spectroscopy. *J Mol Biol* 2001;309:961–974. [PubMed: 11399072]
44. Korzhnev DM, Skrynnikov NR, Millet O, Torchia DA, Kay LE. An NMR experiment for the accurate measurement of heteronuclear spin-lock relaxation rates. *J Am Chem Soc* 2002;124:10743–10753. [PubMed: 12207529]
45. Farrow NA, Zhang O, Szabo A, Torchia DA, Kay LE. Spectral density function mapping using ¹⁵N relaxation data exclusively. *J Biomol NMR* 1995;6:153–162. [PubMed: 8589604]
46. Lefevre JF, Dayie KT, Peng JW, Wagner G. Internal mobility in the partially folded DNA binding and dimerization domains of GAL4: NMR analysis of the N-H spectral density functions. *Biochemistry* 1996;35:2674–2686. [PubMed: 8611573]
47. Bracken C, Carr PA, Cavanagh J, Palmer AG 3rd. Temperature dependence of intramolecular dynamics of the basic leucine zipper of GCN4: implications for the entropy of association with DNA. *J Mol Biol* 1999;285:2133–2146. [PubMed: 9925790]
48. Krizova H, Zidek L, Stone MJ, Novotny MV, Sklenar V. Temperature-dependent spectral density analysis applied to monitoring backbone dynamics of major urinary protein-I complexed with the

pheromone 2- sec-butyl-4,5-dihydrothiazole. J Biomol NMR 2004;28:369–384. [PubMed: 14872128]

Figure 1.

Distinct conformational states of CAP^N. 2D ^1H - ^{15}N HSQC spectra of U- ^2H - ^{15}N -labeled CAP^N in the (a) apo, (b) singly liganded complex (cAMP₁-CAP^N), and (c) doubly liganded complex (cAMP₂-CAP^N). Spectra were recorded at 30 °C on a 600-MHz NMR instrument. Molecular models were drawn using the structure of cAMP₂-CAP (PDB entry 1G6N). cAMP is shown in green sticks.

Figure 2.

Effect of the sequential cAMP binding on the structure of CAP^N assessed by chemical shift mapping. The chemical shift is a highly sensitive probe of the local electronic environment of the nucleus and, thus, provides very useful information about the chemical structure of the protein. The combined chemical shift change of a particular residue upon ligand binding was

calculated as: $\Delta\delta = \sqrt{(\omega_{HN}\Delta\delta_{HN})^2 + (\omega_N\Delta\delta_N)^2 + (\omega_{Ca}\Delta\delta_{Ca})^2 + (\omega_{Cb}\Delta\delta_{Cb})^2 + (\omega_{Co}\Delta\delta_{Co})^2}$, where ω_i denotes the weight factor of nucleus *i*. $\omega_{HN} = 1$, $\omega_N = 0.154$, $\omega_{Ca} = \omega_{Cb} = 0.276$ and $\omega_{Co} = 0.341$ (ref. 43). The color code used to display $\Delta\delta$ (in ppm) is as follows: $\Delta\delta \sim 0$, dark blue; $0.02 < \Delta\delta < 0.08$, light blue; $0.08 < \Delta\delta < 0.2$, magenta; $0.2 < \Delta\delta < 0.4$, red; $0.4 < \Delta\delta < 0.6$, orange; $\Delta\delta > 0.7$, yellow. $\Delta\delta$ for the cAMP₂-CAP^N complex reflects the binding effect of the second cAMP molecule on the cAMP₁-CAP^N complex. Proline residues are colored white.

Figure 3.

Effect of the sequential cAMP binding on the slow motions of CAP^N. **(a)** The conformational exchange dynamics on μ s-ms time scale, as indicated by the term R_{ex} , are mapped on the structure of the protein as a function of the ligation state. Low values indicate rigidity, whereas higher values indicate flexibility. The color code used to represent R_{ex} (in Hz) is as follows: $R_{ex} \sim 0$, dark blue; $1 < R_{ex} < 4$, light blue; $4 < R_{ex} < 8$, magenta; $8 < R_{ex} < 12$, red; $12 < R_{ex} < 16$, orange; $R_{ex} > 16$, yellow. **(b)** Numerical values of R_{ex} for the singly liganded cAMP₁-CAP^N complex plotted as a function of residue number. Values for the liganded and unliganded subunit are shown in red and blue, respectively. Only residues with R_{ex} higher than 2 Hz are displayed. The secondary structure boundaries are according to the X-ray structure of cAMP₂-CAP complex (PDB 1G6N).

Figure 4.

CPMG relaxation dispersion data of ^{15}N backbone amides of CAP^N. **(a)** Representative curves for residues of the liganded subunit of the cAMP₁-CAP^N complex. k_{ex} is $1,100 \pm 320 \text{ s}^{-1}$ for Leu113, $1,350 \pm 290 \text{ s}^{-1}$ for Ile97, $1,200 \pm 250 \text{ s}^{-1}$ for Lys44, and $1,150 \pm 240 \text{ s}^{-1}$ for Tyr41. **(b)** Representative curves for residues of the unliganded subunit of the cAMP₁-CAP^N complex. k_{ex} is $1,100 \pm 330 \text{ s}^{-1}$ for Met120, $1,820 \pm 360 \text{ s}^{-1}$ for Ile106, $1,180 \pm 220 \text{ s}^{-1}$ for Ala95, and $1,3200 \pm 240 \text{ s}^{-1}$ for Leu29.

Figure 5.

Effect of the sequential cAMP binding on the fast motions of CAP^N. **(a)** The amplitude of the motions on ps-ns time scale is indicated by the spectral density function $J(0.87\omega_H)$. The magnitude of $J(0.87\omega_H)$ is mapped in a continuous-scale color onto the structure as a function of the ligation state. Low values indicate rigidity, whereas higher values indicate flexibility. **(b)** Numerical values of $J(0.87\omega_H)$ plotted as a function of residue number. The color code used is as follows: apo-CAP^N, blue; liganded subunit of the cAMP₁-CAP^N complex, green; unliganded subunit of the cAMP₁-CAP^N complex, orange; doubly liganded cAMP₂-CAP^N complex, red.

Figure 6.

Effect of the sequential binding of cAMP to CAP^N on the amide exchange rates. The effect is presented as the ratio of exchange rates k_{ex1}/k_{ex2} induced by each cAMP binding to CAP^N, where k_{ex1} and k_{ex2} refer to the state prior and post cAMP binding, respectively. Lower exchange rates indicate higher protection of the amide hydrogens against exchange with solvent deuterons. Thus, higher values of the k_{ex1}/k_{ex2} ratio indicate decreased protein flexibility and increased protection. Higher protection denotes narrowing of the population of the protein conformational ensemble as conformations from which exchange take place are less populated. Residues with no detectable protection are colored white. Because exchange rates were measured for all states (apo-CAP^N, cAMP₁-CAP^N, and cAMP₂-CAP^N) under identical conditions, k_{ex} values can be directly compared.

Figure 7.

Energetics of the cooperative sequential binding of cAMP to CAP^N. ITC traces (upper panel) and binding isotherm (lower panel) of the calorimetric titration of 0.6 mM cAMP to 30 μM CAP^N at 30 °C. The solid line represents the fit to a sequential binding site model. The individual thermodynamic parameters obtained for the first and second binding step of cAMP are included in the figure. Dissociation constants are 0.04 μM and 4 μM for the first and second cAMP binding step, respectively.

Figure 8.

Effect of the sequential binding of cAMP on the order parameters of CAP^N. The magnitude of the change in the order parameter, ΔS^2 , is mapped in a continuous-scale color onto the structure. Residues with negative ΔS^2 values are colored white. ΔS^2 is given as S^2 (after binding) - S^2 (before binding), so that positive ΔS^2 values denote enhanced rigidity of the protein backbone upon binding. The conformational entropic penalty that accompanies the binding of the second cAMP ($18.1 \text{ kcal mol}^{-1}$) is much larger than the corresponding penalty of the first cAMP binding ($3.2 \text{ kcal mol}^{-1}$). Numerical values of ΔS^2 are provided in Supplementary Figure 5.

Figure 9.

Overall effect of the sequential cAMP binding to the conformation and dynamics of CAP^N. In the absence of cAMP (apo state) CAP^N exhibits considerable flexibility on the ps-ns time scale and limited stability (enhanced H/D exchange rates). The cAMP binding sites are also flexible (denoted by wavy lines). Binding of the first cAMP molecule has little effect on the ps-ns and H/D exchange rates, but it activates the slow motions (μs-ms) of both subunits. While the conformation of the liganded subunit of the cAMP₁-CAP^N complex is extensively affected, the conformation of the unliganded subunit is not perturbed. Subsequent binding of the second cAMP molecule suppresses drastically both slow and fast motions and increases stability (lower H/D exchange rates). The much lower favorable entropy change that accompanies the second cAMP binding over the first one is the source of the negative cooperativity. Red and blue indicate activated and suppressed motions, respectively. A change in the mean conformation is indicated by the overall shape of each subunit.