Thermal Denaturation of the Apo-cyclic AMP Receptor Protein and Noncovalent Interactions between Its Domains

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Cyclic AMP receptor protein (CRP) is allosterically activated by cAMP and functions as a global transcription regulator in enteric bacteria. Structural information on CRP in the absence of cAMP (apo-CRP) is essential to fully understand its allosteric behavior. In this study we demonstrated interdomain interactions in apo-CRP, using a comparative thermodynamic approach to the intact protein and its isolated domains, which were prepared either by limited proteolysis or using recombinant DNA. Thermal denaturation of the intact apo-CRP, monitored by differential scanning calorimetry, revealed an apparently single cooperative transition with a slight asymmetry. Combined with circular dichroism and fluorescence analysis, the thermal denaturation of apo-CRP could be interpreted as a coupled process involving two individual transitions, each attributable to a structural domain. When isolated individually, both of the domains exhibited significantly altered thermal behavior, thus pointing to the existence of non-covalent interdomain interactions in the intact apo-CRP. These observations suggest that the allosteric conformational change of CRP upon binding to cAMP is achieved by perturbing or modifying pre-existing interdomain interactions. They also underline the effectiveness of a comparative approach using calorimetric and structural probes for studying the thermodynamics of a protein.

INTRODUCTION

Cyclic AMP receptor protein (CRP) plays a key role in transcription regulation at nearly 200 different promoters in prokaryotes (Hollands et al., 2007), by interacting with RNA polymerase, as well as by binding to specific DNA sites. The protein is inactive in its apo form, i.e., in the absence of cAMP, but it adopts an active conformation upon binding its effector molecule, cAMP (Botsford and Harman, 1992; Harman, 2001; Kolb et al., 1993; Lawson et al., 2004). X-ray crystallographic studies have contributed to a comprehensive understanding of the protein structure in its three functional states: CRP-cAMP (Chu et al., 2001; Passner et al., 2000; Weber and Steitz, 1987), CRP-cAMP-DNA (Parkinson et al., 1996; Passner and Steitz, 1997; Schultz et al., 1991), and CRP-cAMP-DNA-RNA polymerase (Benoff et al., 2002). Up to now, approximately twenty coordinates for the 3-dimensional structure of CRP have been deposited in the Protein Data Bank. Nevertheless the structure of the unliganded form (apo-CRP), which is essential to understand the activation process, has not yet been solved.

Escherichia coli CRP is a homodimeric protein with 209 amino acids in each subunit. In the known crystal structures, each subunit of CRP is folded into two structurally distinct domains, which are covalently connected by a short stretch of amino acids, named the hinge region (residues 135–138), running between the C-helix of the N-terminal domain and the D-helix of the C-terminal domain. The larger N-terminal domain is responsible for the intersubunit contacts for dimerization and provides the cAMP binding specificity through a β-roll structure. The smaller C-terminal domain is involved in specific recognition of DNA via a helix-turn-helix motif. Both the domains can interact with RNA polymerase (Lawson et al., 2004) through three sites named activating regions (ARs). The C-terminal domain possesses AR1 (residues 154–164), which interacts with RNA polymerase at CRP-dependent class I and class II promoters. AR1 can mediate a physiologically relevant contact with the C-terminal domain of the RNA polymerase β-subunit, even in the absence of promoter DNA (Heyduk et al., 1993; Lee et al., 2001). At CRP-dependent class II promoters the interaction with RNA polymerase is complemented by the other two activating regions located in the N-terminal domain of CRP: AR2 (residues 19, 21, 96, and 101) and AR3 (residues 52–55, 58). AR2 contacts the N-terminal domain of the β-subunit and AR3 interacts with the sigma factor (σ70) of RNA polymerase.

Since the isolation of CRP in 1970, a variety of biochemical and biophysical studies have indicated that, upon cAMP binding, CRP undergoes allosteric conformational changes to assume an active conformation (Harman 2001; Won et al., 2000; 2002), in which allosteric signals are transmitted from the N-terminal to the...
C-terminal domain, probably by interdomain communication. The biological functions of many proteins with more than one domain are regulated by interdomain interactions. Thus, it is important to know whether the structural domains in a protein act independently or affect the activity of the protein by interacting with each other. Although it has been generally accepted that the allosteric activation of CRP is a process involving rigid-body movements leading to subunit realignment and domain reorientation (Harman 2001; Won et al., 2000, 2002), most research has focused on the inter-subunit communication. In addition, features of the interdomain interactions in CRP have been established only for its cAMP-bound state, since the apo-CRP structure was not available. Consequently, it was not clear whether the interdomain networks observed in cAMP-bound CRP were generated upon binding to cAMP or were modified from interdomain interactions pre-existing in apo-CRP.

Structural information about apo-CRP, particularly concerning its interdomain communication, is a fundamental requisite for understanding CRP allostery. The present study provides thermodynamic data that establish the occurrence of an interdomain interaction in apo-CRP. To interpret the complicated thermal behavior of apo-CRP, we employed spectroscopic methods that probe the protein structure in different ways, together with calorimetric measurements. The data enabled us to perform a qualitative or semi-quantitative comparison of the thermodynamic properties of the intact apo-CRP and its separate domains. We anticipate that our results will facilitate further biophysical investigations of the structure and interactions of the apo-CRP domains in solution.

MATERIALS AND METHODS

Protein sample preparation

Recombinant E. coli CRP was prepared from the overproducing E. coli strain BL21(DE3)/pLyS containing the plasmid pT7-CRP, as reported previously (Lee et al., 2001; Won et al., 2000). To produce recombinant βCRP, DNA fragments encoding amino acid residues 110–209 of CRP were amplified by PCR with plasmid pT7-CRP, as template, and cloned into the pET-21a vector, followed by transformation into E. coli BL21 (DE3)/pLyS. The oligonucleotide primers contained stop codons to produce the protein, without artificial histidine tags. The cells expressing CRP or βCRP were grown at 37°C in M9 minimal medium and protein expression was induced by adding IPTG (Sigma). Protein purification was performed by sequential chromatography on Bio-Rex 70 (Bio-Rad), Blue Sepharose (Amersham), Hydroxyapatite (Bio-Rad), and Superdex 75 (Pharmacia). ChuyCRP was prepared by limited proteolysis of the purified CRP with chymotrypsin, according to the method by Blaszczzyk and Wasylewski (2003) with some modifications. A solution of 1.5 mM motrypsin, according to the method by Blaszczyk and Wasyl-
RESULTS AND DISCUSSION

DSC-monitored thermal denaturation of apo-CRP
We first monitored the thermal denaturation of apo-CRP, using differential scanning calorimetry (DSC), to test for a possible interdomain interaction (Fig. 1). Comprehensive thermodynamic studies of CRP have been previously undertaken by Ghosaini et al. (1998) and Blaszczyk and Wasylewski (2003), to determine the energetics of CRP interaction with cyclic nucleotides and DNA. The DSC profiles of CRP were characterized by multiple transitions in the presence of cAMP, and an apparently single transition in the absence of cAMP. In our measurement, thermal denaturation of CRP exhibited an irreversible endothermic transition, consistent with the previous observations. The present DSC profile of apo-CRP is also in good agreement with those in the previous reports. In particular, the thermodynamic parameters derived from the present data ($T_c = 65.1 \pm 0.1^\circ C$, $\Delta H_c = 130.0 \pm 8.8$ kcal/mol) were more consistent with those reported by Ghosaini et al. (1998) ($T_c = 64.4 \pm 0.1^\circ C$, $\Delta H_c = 130.7 \pm 6.0$ kcal/mol), whose buffer conditions were similar to ours, than to those reported by Blaszczyk and Wasylewski (2003) ($T_c = 62.5 \pm 0.1^\circ C$, $\Delta H_c = 142.5 \pm 3.5$ kcal/mol).

Although the previous studies treated the thermal denaturation of apo-CRP as a single cooperative transition, we noted that the transition was asymmetric and approached a deconvolution into two non-two-state curves, which is applicable to irreversible transitions. Figure 1 clearly shows that the denaturation curve of apo-CRP could be decomposed into two overlapping individual transitions (or calorimetric domains): $T_c = 64.0 \pm 0.2^\circ C$ and $\Delta H_c = 50.9 \pm 8.8$ kcal/mol for the first transition, and $T_c = 65.3 \pm 0.1^\circ C$ and $\Delta H_c = 80.0 \pm 8.7$ kcal/mol for the second transition. The DSC profile of a protein is often deconvoluted into multiple components that represent separate transitions of individual domains (Blandamer et al., 1994; den Blauwen et al., 1999; Pabo et al., 1979; Privalov, 1982; Wenk et al., 1998; Zaiss and Jaenicke, 1999). Thus, we assumed that the two components in Fig. 1 might be attributable to separate transitions of the domains of apo-CRP. To check this possibility, thermal denaturation of apo-CRP was further investigated by circular dichroism (CD) and fluorescence spectroscopy.

Spectroscopic monitoring of apo-CRP denaturation
Generally, far-UV CD spectra monitor the secondary structure of proteins, and the signals at 222 nm, in particular, are dominated by $\alpha$-helices (Eun et al., 2006; Rodger and Nordén, 1997; Sreerama and Woody, 2000; Won et al., 2004a, 2004b). Thus, the CD-monitored profile in Fig. 2 could reflect denaturation of the whole molecule and be attributed to the C-terminal domain, which consists mostly of $\alpha$-helices, rather than to the N-terminal domain, which is mainly populated by $\beta$-sheets. In contrast, the intrinsic fluorescence of proteins reflects the microenvironment of aromatic side-chains, and fluorescence emissions at an excitation wavelength of 280 nm are dominated by tryptophan residues. Since CRP possesses two tryptophan residues and these are in the N-terminal domain, the fluorescence-monitored profile in Fig. 2 could specifically represent the thermal denaturation of the N-terminal domain. If apo-CRP behaves as a single calorimetric domain, the two profiles are expected to be mutually consistent (Byrne and Stites, 2007). However, the denaturation curve obtained from fluorescence measurements ($T_c = 62.9^\circ C$) preceded the CD profile ($T_c = 66.1^\circ C$), by more than 3°C. Based on the temperature ranges of denaturation and the $T_c$ values, it seems that the fluorescence and CD curves correspond to deconvoluted transitions 1 and 2, respectively, in the DSC profile in Fig. 1.

In summary, combining the spectroscopic and calorimetric analyses, the present results suggest that thermal denaturation of apo-CRP occurs separately in its individual domains, even though their transitions are probably strongly coupled. The transition of the N-terminal domain, which can be assigned to the fluorescence trace and deconvoluted DSC curve 1, occurs at a rather lower temperature than the C-terminal domain denaturation that can be assigned to the CD trace and deconvoluted DSC curve 2. To examine the possible interaction between the two domains in apo-CRP, we analyzed the thermal behavior of each domain on its own, in comparison with that of the intact apo-CRP.

Domain constructs of CRP
Apo-CRP dimer is resistant to proteolysis, but the protein can be cleaved with a number of proteolytic enzymes in the presence of cAMP, producing dimers of the N-terminal domain (Angulo and Krakow, 1985). These fragments, named c,CRP, retain cAMP binding ability and a structure comparable to that of the corresponding regions of intact CRP (Clore and Gronenborn, 1982; Li et al., 2002; Popovych et al., 2006). In particular, chymotrypsin digestion of CRP produces a fragment consisting of residues 1–136 (designated CHxCRP), which preserves the
Fig. 3. Thermal denaturation of apo-CHβCRP monitored by CD (filled circles) and fluorescence (empty triangles) spectroscopy. The CD-monitored denaturation profile of apo-CRP is shown for comparison (empty circles). The constructed regions are indicated by the ribbon representation in the crystal structure of the cAMP-bound CRP (PDB entry 1G6N). The denatured fraction \( F_d \) was calculated from the CD signals at 222 nm and the fluorescence emission ratio at \( \lambda_{	ext{max}} \) (344 nm) and \( \lambda_{	ext{exc}} \) (336 nm), respectively (refer to the text for details).

Fig. 4. Thermal denaturation of βCRP monitored by CD spectroscopy (filled circles). The CD-monitored denaturation profile of apo-CRP is shown for comparison (empty circles). The constructed regions are indicated by a ribbon representation in the crystal structure of the cAMP-bound CRP (PDB entry 1G6N). The denatured fraction \( F_d \) was calculated from the CD signals at 222 nm (refer to the text for details).

Thermal denaturation of CRP domains

Many examples of domain isolation result in changes of thermodynamic properties (Blandamer et al., 1994; Privalov, 1982; Wenk et al., 1998; Zais and Jaenicke, 1999). Even if the domain conformation is not changed, its isolation can result in dramatic alterations of its thermodynamic and/or kinetic stability, due to the loss of noncovalent interdomain interaction occurring in the intact protein. In contrast, independent domains not interacting with each other in the intact protein do not change their thermodynamic behavior when the covalent linkage between them is broken (den Blaauwen et al., 1999; Lee et al., 2007; Pabo et al., 1979; Privalov, 1982). The results in Figs. 3 and 4 demonstrate the existence of a strong interdomain interaction in apo-CRP, since the denaturation profiles of apo-CHβCRP and βCRP differ significantly from that of the intact apo-CRP. Based on the CD profiles, apo-CHβCRP exhibited a \( T_m \) approximately 5°C higher than that of apo-CRP, with a slightly broadened range of denaturing temperature (Fig. 3). The fluorescence-monitored denaturation of apo-CHβCRP \( (T_m = 70.8°C) \) was in good agreement with that obtained by CD \( (T_m = 71.3°C) \), suggesting that the protein behaves as a single calorimetric unit. The slight differences are attributable to the systematic differences (sensitivity, noise level, resolution, baseline linearity, and etc.) between the two probes of structure, fluorescence and CD, and/or a non-two-state transition of the protein (Byrne and Stites, 2007). Thus, from the N-terminal domain transition in apo-CRP (DSC curve 1 in Fig. 1 and fluorescence curve in Fig. 2), it can be concluded that isolation of the N-terminal domain increased its \( T_m \) by more than 7°C. The denaturation of βCRP, monitored by CD, was quite different from that of the intact apo-CRP (Fig. 4). The \( T_m \) of βCRP \( (T_m = 71.1°C) \) was similar to that of the apo-CHβCRP, showing a significant increase compared to that of the intact apo-CRP. Compared with the C-terminal domain transition in apo-CRP (DSC curve 2 in Fig. 1 and CD curve in Fig. 2), isolation of the C-terminal domain increased its \( T_m \) by more than 5°C. However, the temperature range of denaturation was more than 4-fold broader than that of apo-CRP or apo-CHβCRP, indicating a significant decrease of cooperativity of the transition. This observation is consistent with the H-D exchange results of Li et al. (2002), which suggested that the βCRP conformation was very dynamic and/or rather loose.

In summary, the thermal behavior of the isolated domains, characterized by a significant increase of \( T_m \) and a decreased cooperativity, especially in the case of the C-terminal domain, was quite different from that of the intact apo-CRP. As noted above, the conformation of the present domain constructs is probably comparable to that of the corresponding regions in the intact apo-CRP; i.e., the conformations of the two domains are not significantly perturbed by breaking the covalent linkage between them. Thus, the dramatic alteration of the denaturing properties of the individual domains from the intact forms to the isolated forms, is not attributable to any conformational change but results from the loss of noncovalent interactions between them.

Concluding remarks

In this work, the thermal denaturation of apo-CRP, which has been regarded as a single cooperative transition, is reinterpreted as the result of overlap of the individual transitions of the individual structural domains. Our results also show how spectroscopic methods such as circular dichroism and fluorescence can be combined with the calorimetric approach to define the thermodynamic behavior of proteins from a structural


