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Scanning Calorimetric Study of the Thermal Unfolding of Catabolite Activator Protein from *Escherichia coli* in the Absence and Presence of Cyclic Mononucleotides†

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ABSTRACT: The thermal unfolding of the catabolite activator protein (CAP) of *Escherichia coli* and the complexes it forms with adenosine cyclic 3′,5′-phosphate (cAMP) and guanosine cyclic 3′,5′-phosphate (cGMP) was studied by high-sensitivity differential scanning calorimetry (DSC). The thermal denaturation of CAP at pH 7.00 gave an irreversible, symmetrical denaturation curve with a single peak. Distinctly different, more complex DSC curves were obtained for the thermal denaturation of the cAMP–protein and cGMP–protein complexes. The DSC data indicate intermolecular cooperation among CAP dimers, with the extent of oligomerization remaining unchanged during unfolding of the protein. The DSC curves for the thermal denaturation of the cAMP–protein complex and cGMP–protein complex have been resolved into three and two components, respectively, according to the model of independent two-state processes. Analysis of the DSC data suggests two and three independent domains for cGMP–protein and cAMP–protein complexes, respectively, with dissociation of mononucleotide occurring in the second component in both cases during protein denaturation. Furthermore, our studies indicate that the presence of either ligand alters the degree of oligomerization of CAP dimers, cAMP having a greater effect than cGMP.

The catabolite activator protein (CAP)[1] or cAMP receptor protein (CRP) of *Escherichia coli* is a well-characterized ligand-induced regulatory protein. The protein complexed with cAMP binds to specific DNA sequences near promoter regions and stimulates transcription (de Crombrugghe et al., 1984).

CAP is a dimeric protein composed of two identical subunits, 22 500 daltons each (Anderson et al., 1971). X-ray crystallographic studies (McKay & Steitz, 1981; McKay et al., 1982) of the cAMP–protein complex to 2.9-Å resolution show that each subunit contains two domains connected by a single covalent stretch of polypeptide: an NH_2-terminal domain that binds cAMP and provides all subunit–subunit contacts and a COOH-terminal domain that binds DNA (Krakow & Pastan, 1972; Eilen et al., 1978). A comprehensive study undertaken by Ebright et al. (1985) involving cAMP, cGMP, and various cAMP analogues reveals changes elicited in the structure of CAP upon nucleotide binding. A greater susceptibility to trypsin cleavage (Krakow & Pastan, 1973) and intersubunit disulfide cross-linking (Eilen & Krakow, 1977) results upon cAMP binding, properties not affected by cGMP. This has been interpreted as indicating that the binding of cAMP to CAP protein elicits a conformational change in the dimer structure while cGMP, which does not stimulate specific DNA binding and transcription, induces no biochemically detected conformational change. The importance of CAP and its complex with cAMP in the transcriptional regulation of some 25 genes in *E. coli* prompted an investigation of the thermodynamics of unfolding of this protein in the absence and presence of either of the ligands, cAMP or cGMP. The isolation and purification of relatively large quantities of CAP protein by a recently developed procedure (Brown, 1987) made possible a study by means of high-sensitivity differential scanning calorimetry. We report here on the thermal unfolding of CAP protein and its complexes with cAMP and cGMP.

Materials and Methods

CAP was isolated from *E. coli* strain HB101 harboring plasmid pHW, a generous gift from Dr. H. M. Wu. The purification and concentration of CAP were carried out on a large scale in a single step by affinity chromatography on cAMP–agarose. Typically, 60 g of cells was suspended in 300 mL of lysis buffer (20 mM sodium phosphate, 20 mM NaCl,

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[1] Abbreviations: cAMP, adenosine cyclic 3′,5′-phosphate; CAP, catabolite activator protein; CRP, cyclic AMP receptor protein (another term for CAP); DSC, differential scanning calorimetry; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; cGMP, guanosine cyclic 3′,5′-phosphate.

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Table I: Thermodynamic Data for the Thermal Unfolding of CAP Protein at pH 7.0

protein concn (μM)	$t_{1/2}$ (°C)	ΔH (kcal mol^{-1})	ΔH^a (kcal mol^{-1})	ΔH_{vH}^b (kcal mol^{-1})	ΔH_{vH}^c (kcal mol^{-1})	$\Delta H_{vH}/\Delta H$	ΔC_p (kcal K^{-1} mol^{-1})	SD, % of $C_{ex,max}$
23.3–160.2	66.4 ± 0.1[d]	130.7 ± 6.0[d]	130.5 ± 5.2[d]	231 ± 9[d]	230 ± 8[d]	1.8 ± 0.1[d]	0.42 ± 0.43[d]	4.3 ± 0.4[d]

[a] ΔH obtained by planimeter integration. [b] Evaluated as $\Delta H_{vH} = \beta\Delta h_{cal}$. [c] Evaluated from $\Delta H_{vH} = 4RT_{1/2}^2C_{ex,1/2}/\Delta h_{cal}$. [d] Standard error of the mean values.

2 mM EDTA, 5 mM β-mercaptoethanol, and 5% glycerol) at pH 7.2 and lysed in a French pressure cell at >10 000 psi. The volume of buffer was then doubled and adjusted to 40% saturated ammonium sulfate and stirred for 30 min at 4 °C. Following centrifugation at 14000g for 1 h, the supernatant was readjusted to 70% saturation with ammonium sulfate and stirred for 1 h and the centrifugation repeated. The pellet was suspended in 20 mL of buffer A (200 mM sodium phosphate, 2 mM EDTA, 5 mM β-mercaptoethanol, and 5% glycerol) with 0.5 M NaCl added at pH 7.2 and dialyzed against the same buffer for 2 h and then with two changes of buffer A overnight. A 2.5 × 20 cm column containing 100 mL of cAMP–agarose was prewashed with buffer A + 1 M NaCl and then equilibrated with buffer A. Following centrifugation at 14000g for 30 min, the cleared dialysate was applied to the column at a flow rate of 20 mL/h. The column was then washed with buffer A + 300 mM NaCl at a flow rate of 60–90 mL/h until the ratio of OD_{280}/OD_{260} equaled or exceeded 1.5. Only CAP preparations with a final OD_{280}/OD_{260} ratio exceeding 1.5 gave a single transition in our DSC experiments. Elution of purified CAP protein was carried out at a flow rate of 10 mL/H with buffer A containing 0.5 M NaCl and 5 mM cAMP. The elution profile was followed by optical absorption at 295 nm, where absorption by cAMP is negligible. Peak protein concentrations exceeding 15 mg mL^{-1} were obtained of purity >97% as determined by gel electrophoresis. The cyclic nucleotide was removed by rapid filtration over coarse Sephadex G-50 in a second 2.5 × 45 cm column equilibrated with buffer A containing 0.5 M NaCl at a flow rate of 3 mL min^{-1}. The effluent was collected in fractions and the absorption monitored at 280 nm. Pooled fractions were dialyzed overnight against 50 mM potassium phosphate containing 0.5 M KCl, 0.2 mM EDTA, 0.2 mM DTT, and 5% glycerol at pH 7.0 and were stored in the same buffer at 4 °C. The residual cAMP concentration is estimated to be less than 10% of the CAP concentration, and under these conditions, the protein was found to be stable for at least 1 year. Typically, 60 g of dry cells yielded 0.5 g of purified, nucleotide-free CAP protein. Prior to use, the protein was dialyzed against the same buffer for at least 24 h at 4 °C. Similar dialyses of protein in the appropriate buffers at various pHs were performed for all DSC studies undertaken. The concentration of protein was determined spectrophotometrically by using a Cary 219 spectrometer and an extinction coefficient of $E_{280} = 3.5 \times 10^4$ M^{-1} cm^{-1} (Fried & Crothers, 1984). Since CAP protein is not very soluble at low ionic strength and tends to precipitate on addition of cyclic nucleotides, we have used a buffer of high ionic strength in all our experiments. Our results indicate, as discussed later, that under these circumstances the protein contains to a small extent oligomers higher than the dimer.

cAMP and cGMP were purchased from Sigma and used without further purification. Agarose–hexane–adenosine cyclic 3′,5′-phosphate type 3 (AGcAMP) was from Pharmacia P-L Biochemicals. Ammonium sulfate (ultrapure) was from Schwarz/Mann. All other chemicals were of reagent grade.

The DASM-4 microcalorimeter (Privalov, 1980) was employed in all our DSC experiments. A scan rate of 1 K min^{-1} was used throughout. The effective cell volumes over the appropriate temperature range were determined by measuring the total heat capacities of the cells filled with water and then nitrogen, using the known specific heats and densities of water and nitrogen. For obtaining the thermodynamic parameters describing each DSC curve, the procedures outlined by Privalov and Kchechinashvili (1974) were followed. Base lines were drawn as outlined by Takahashi and Sturtevant (1981).

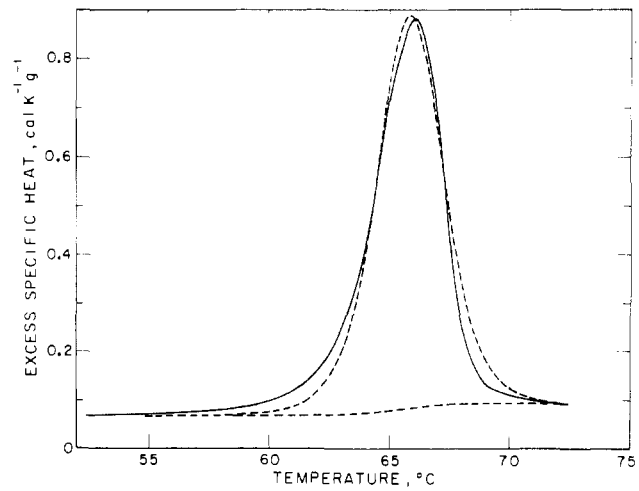

FIGURE 1: Typical DSC curve observed with CAP protein in 50 mM potassium phosphate at pH 7.0. Protein concentration, 2.38 mg mL^{-1} (52.9 μM); scan rate, 1 K min^{-1}. Curve fitting to a two-state model. (- - -) Calculated curve; (—) observed curve.

Results and Discussion

Thermal Denaturation of CAP in the Absence of Cyclic Mononucleotides. The thermal unfolding of CAP was studied at pH 7.0 over the protein concentration range 1–7 mg mL^{-1}. A typical DSC trace, after subtraction of the instrumental base line, for the denaturation process in the absence of added ligand is presented in Figure 1. A single irreversible endothermic peak was obtained under all conditions employed. Denaturation experiments performed using various buffers over a pH range 3–10 and different KCl concentrations (0–0.5 M) failed to give an endotherm which showed reversibility on rescanning after an initial heating. All the relevant DSC data are summarized in Table I, with uncertainties given as the standard errors of the means.

The data obtained in these experiments, and in those to be reported below, showed a larger variability than is usual in recently reported DSC results on protein denaturation. For example, the standard error of the mean in the 14 values for ΔH amounted to 6.0 kcal mol^{-1}, and the mean standard deviation in the curve fitting was 4.3%. This variability is probably due in large measure to difficulty in preparing highly reproducible samples of protein. The lack of reproducibility was especially evident in the pre- and posttransitional base lines, errors which can have large effects because of the long extrapolations involved in using them in the calculations. The unusually large variability in the values for ΔC_p is further indication of these base-line problems. We believe, however, that the large uncertainties in the quantitative aspects of the data do not invalidate our qualitative conclusions.

Table II: Parameters Determined in the Curve Resolution of Experiments with CAP Protein plus cAMP (Protein Concentration 60.2–63.6 μM)

ligand concn (mM)	component 1 $t_{1/2}$ (°C)	component 1 ΔH (kcal mol^{-1})	component 1 ΔH_{vH} (kcal mol^{-1})	component 2 $t_{1/2}$ (°C)	component 2 ΔH (kcal mol^{-1})	component 2 ΔH_{vH} (kcal mol^{-1})	component 3 $t_{1/2}$ (°C)	component 3 ΔH (kcal mol$_{-1}$)	component 3 ΔH_{vH} (kcal mol^{-1})	SD, % of $C_{ex,max}$	ΔC_p[a] (kcal mol^{-1} K^{-1})	$\sum \Delta H$[b] (kcal mol^{-1})	ΔH_{obsd}[c] (kcal mol^{-1})
0.17–12.47	67.1–70.8	36.6 ± 1.6	110 ± 3	71.5–82.6	63.0 ± 2.1	190 ± 3	75.6–89.4	34.0 ± 2.5	114 ± 6	4.0 ± 0.3	1.60 ± 0.54	137.6 ± 5.4	144.2 ± 7.0

[a] The mean value for the overall ΔC_p. [b] The sum of ΔH's of component curves 1, 2, and 3. [c] ΔH obtained by planimeter integration.

There is little change in $t_{1/2}$, the temperature of half-denaturation, over the protein concentration range 1–7 mg mL^{-1}, indicating that the degrees of oligomerization of the native and denatured proteins are approximately the same. Resolution by procedures outlined elsewhere (Sturtevant, 1987) of the DSC curves for CAP, according to a model based on independent two-state steps, showed a best fit of the observed data on the assumption of a single two-state process. The adjustable parameters for each two-state component are $t_{1/2}$, Δh_{cal}, the specific enthalpy at $t_{1/2}$, with the permanent change in apparent specific heat taken into account and $\beta = \Delta H_{vH}/\Delta H_{cal}$ where ΔH_{vH} is the van't Hoff enthalpy. β is assumed to be independent of temperature. The mean values for $\Delta H = M\Delta h_{cal}$ (M = the dimer molecular weight, 45 000) and ΔH_{vH} evaluated by curve resolution are given in columns 3 and 5 of Table I. Column 4 lists the mean calorimetric enthalpy obtained by planimeter integration of the observed DSC curves after deducting the instrumental base line. Agreement between the two sets of values for ΔH is very good. The van't Hoff enthalpies given in column 7 were calculated according to the expression:

$$\Delta H_{vH} = ART_{1/2}^2 C_{ex,1/2}/\Delta h_{cal} \quad (1)$$

where $C_{ex,1/2}$ is the excess over the base line value of the apparent specific heat at $t_{1/2}$, $T_{1/2} = t_{1/2} + 273.15$, and $A = 4.0$ for a single two-state process involving neither association nor dissociation. The reader is referred to previous publications for a justification of the validity of the application of eq 1 to an apparently irreversible denaturation process (Manly et al., 1985; Edge et al., 1985). The fact that the ratio $\Delta H_{vH}/\Delta H$ has a mean value of 1.8, with ΔH calculated on the basis of the dimer molecular weight, indicates a degree of oligomerization approaching tetramerization, which is apparently independent of protein concentration over the range of concentrations studied. The enthalpies and ΔC_p values showed no significant correlation with protein concentration. The overall mean value of the denaturation enthalpy change is 130.7 ± 6.0 kcal mol^{-1}, and that for ΔC_p is 0.42 ± 0.43 kcal K^{-1} mol^{-1} (Table I). In view of the small mean value of ΔC_p and the large uncertainty in its value, we have not calculated the individual values of the denaturation enthalpy to a reference temperature.

Thermal Denaturation of CAP Protein in the Presence of Cyclic Mononucleotides (cAMP and cGMP). Figures 2 and 3 show typical DSC curves obtained for the thermal denaturation of CAP protein in the presence of cAMP and cGMP, respectively, after subtraction of the instrumental base line. It is interesting to note that in contrast to a single peak obtained for the thermal denaturation of the protein alone, in the presence of cAMP or cGMP (at stoichiometric or higher concentrations) multipeaked denaturation curves are observed. This necessitates the decomposition of the observed DSC curves into two or more component curves. All the DSC curves were resolved into component curves according to the model of independent domains unfolding in two-state steps. Although it is obvious that the cooperative nature of most protein structures will ensure that truly independent domains will very

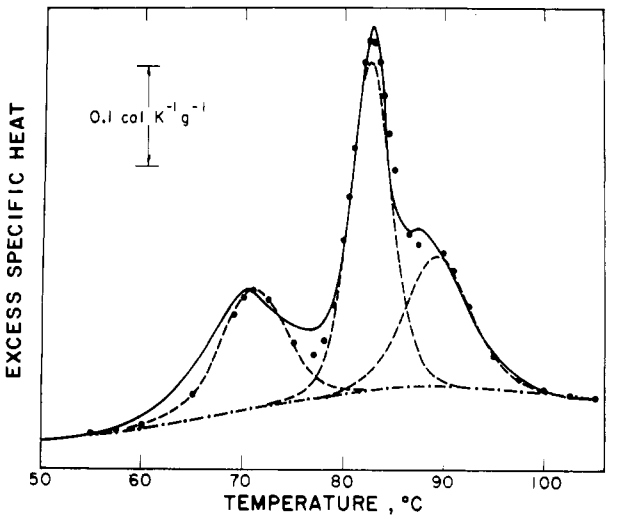

FIGURE 2: Resolution of a DSC curve for cAMP–protein complex into three independent two-state curves. (---) Component curves; (●) sum of component contributions; (—) observed curve. Protein concentration, 2.86 mg mL^{-1} (63.6 μM); cAMP concentration, 12.5 mM; scan rate, 1 K min^{-1}.

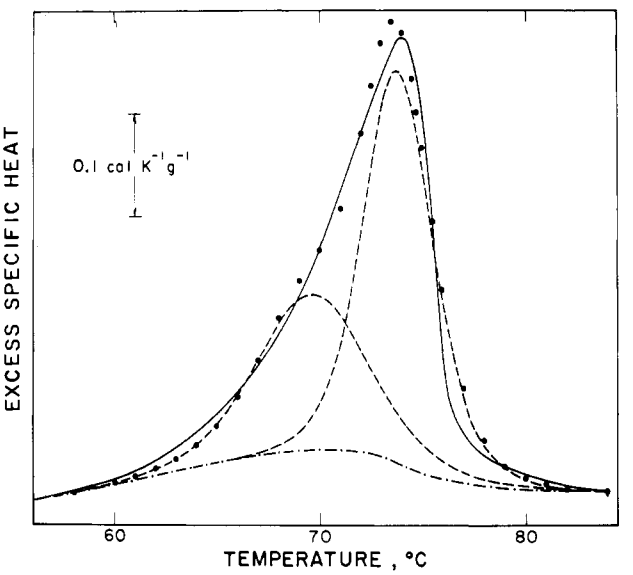

FIGURE 3: Resolution of a DSC curve for cGMP–protein complex into two independent two-state curves. (---) Component curves; (●) sum of component contributions; (—) observed curve. Protein concentration, 2.57 mg mL^{-1} (57.1 μM); cGMP concentration, 5 mM; scan rate, 1 K min^{-1}.

rarely if ever be encountered, we know of no general way to include domain interactions in a model. Resolution of the DSC curves for the thermal unfolding of CAP protein in the presence of cAMP showed that three steps were required to represent the curves, whereas for those obtained in the presence of cGMP, two steps sufficed. Typical curve resolutions are shown in Figure 2 for the thermal unfolding of the protein in the presence of cAMP, and in Figure 3 in the presence of cGMP. The results of the curve resolutions are summarized

Table III: Parameters Determined in the Curve Resolution of Experiments with CAP Protein plus cGMP (Protein Concentration 52.9–60.2 μM)

ligand concn (mM)	component 1 $t_{1/2}$ (°C)	component 1 ΔH (kcal mol^{-1})	component 1 ΔH_{vH} (kcal mol^{-1})	component 2 $t_{1/2}$ (°C)	component 2 ΔH (kcal mol^{-1})	component 2 ΔH_{vH} (kcal mol^{-1})	SD, % of $C_{ex,max}$	ΔC_p[a] (kcal K^{-1} mol^{-1})	$\sum \Delta H$[b] (kcal mol^{-1})	ΔH_{obsd}[c] (kcal mol^{-1})	$\Delta H_{vH}/\Delta H$
0.17–15.0	64.7–71.8	50.7 ± 1.9	129 ± 6	68.6–76.8	90.8 ± 3.9	231 ± 10	3.9 ± 0.3	0.73 ± 0.66	141.9 ± 4.7	142.2 ± 4.7	2.6 ± 0.2

[a] The mean value for the overall ΔC_p. [b] The sum of ΔH's of component curves 1 and 2. [c] ΔH obtained by planimeter integration.

Table IV: van't Hoff Enthalpies for the Unfolding of CAP Protein in the Presence of cAMP

component	ΔH_{vH} (kcal mol^{-1}) curve fitting	ΔH_{vH} (kcal mol^{-1}) van't Hoff plot	coeff of determination
1	110 ± 3	(236)	0.63
2	190 ± 3	97	0.99
3	114 ± 6	85	0.98

Table V: van't Hoff Enthalpies for the Unfolding of CAP Protein in the Presence of cGMP

component	ΔH_{vH} (kcal mol^{-1}) curve fitting	ΔH_{vH} (kcal mol^{-1}) van't Hoff plot	coeff of determination
1	129 ± 6	134	0.76
2	231 ± 10	117	0.91

in Table II for CAP plus cAMP and in Table III for CAP plus cGMP. In each case, the results obtained for the mean values or ranges of values of the various parameters are listed. For each component, the three fitting parameters, $t_{1/2}$, ΔH, and ΔH_{vH}, are listed. Also given are the mean standard deviations of the calculated from the observed excess specific heats, expressed as a percent of the maximal value of the excess specific heat.

Some support for the curve resolutions is given by comparisons of the sum of the ΔH values for each component and the total enthalpy obtained by planimeter integration of the experimental DSC curves, and of the values of ΔH_{vH} obtained by curve resolution and by the application of eq 1. As seen in Tables II and III, the sum of the enthalpies of the component curves in the presence of either ligand agrees well with the total, ΔH_{obsd}, as evaluated by planimeter integration of the DSC curves. Similarly, the values of ΔH_{vH} obtained by curve resolution agree very well with those evaluated according to eq 1 (data not shown in tables).

One effect of increasing concentrations of cAMP and cGMP on the thermal denaturation of CAP protein was to increase the value of $t_{1/2}$ for each composite peak. This effect of added ligands is shown in Figures 4 and 5 in the form of van't Hoff plots of ln $[L_0]$ vs $1/T_{1/2}$ where $[L_0]$ is the total ligand concentration [cf. Fukada et al. (1983)]. Slopes of the van't Hoff plots for the second and third components in the case of cAMP, with coefficients of determination of 0.99 and 0.98, respectively, show approximate equality of these values. Multiplication of the slopes by $-R$, the negative of the gas constant, gives 236, 97, and 85 kcal mol^{-1} for components 1, 2, and 3, respectively. These van't Hoff enthalpies derived from the plots are listed in Tables IV and V for the cases of cAMP and cGMP, respectively. Also listed are van't Hoff enthalpies obtained by curve resolution (column 2).

In attempting to interpret these data for the case of cAMP, it is evident that no more than two of these slopes can be interpreted as yielding van't Hoff enthalpies since only two molecules of cAMP are bound per dimeric CAP. We have disregarded the slope for component 1 in view of the very poor coefficient of determination. It can be shown by means of computer simulations that in the case of a multidomain protein with domain interactions, it is possible for the value of $t_{1/2}$ for

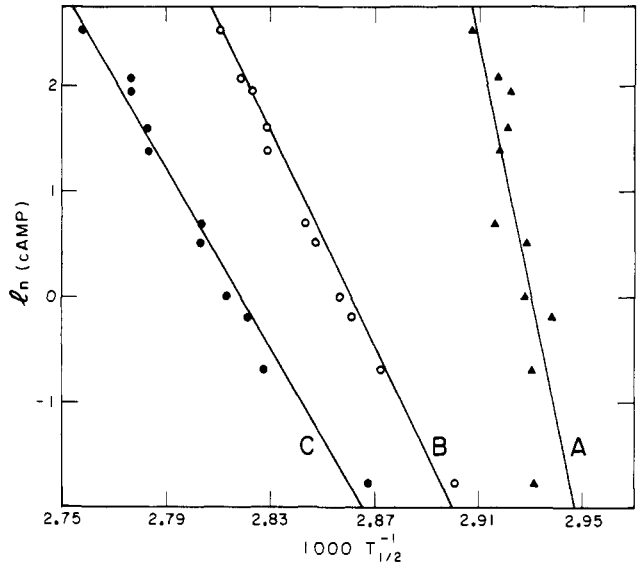

FIGURE 4: van't Hoff plots of the logarithm of the total concentration of cAMP vs $1000/T_{1/2}$ for independent steps 1, 2, and 3 (plots A, B, and C, respectively) obtained by curve resolution of experiments in the presence of cAMP; concentrations of 0.17–12.5 mM.

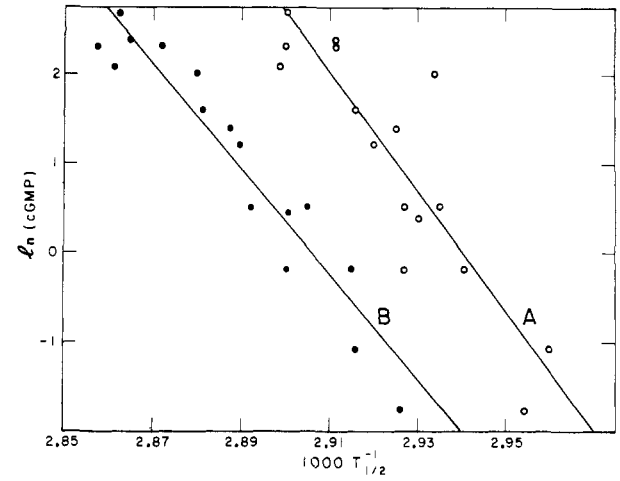

FIGURE 5: van't Hoff plots of the logarithm of the total concentration of cGMP vs $1000/T_{1/2}$ for independent steps 1 and 2 (plots A and B, respectively) obtained by curve resolution of experiments in the presence of cGMP; concentrations of 0.17–15.0 mM.

the unfolding of a domain to which no ligand is bound to change with ligand concentration in much the same manner as $t_{1/2}$ of a domain from which ligand dissociates on denaturation. Since $\Delta H_{vH} = -nRS$ where S is the slope of the van't Hoff plot and n is the number of moles of ligand dissociated per step in the denaturation, it might be suggested on the basis of the values listed in Table IV that 2 mol of ligand is dissociated during denaturation of component 2 and that the slope of the line for component 3 arises from domain interactions, although dissociation of mononucleotide during component 3 cannot be entirely ruled out.

The slopes of the van't Hoff plots in Figure 5 give apparent van't Hoff enthalpies for the case of cGMP of 134 and 117

kcal mol^{-1} for components 1 and 2, respectively. No clearcut interpretation of these values is evident. We incline to the view that both bound molecules of cGMP are dissociated during denaturation of component 2, so that the expected value of ΔH_{vH} is approximately 2 × 117 kcal mol^{-1}, in good agreement with the value obtained from curve fitting, and that the slope of the line for component 1 arises from domain interactions. It appears that under the conditions of our experiments CAP protein has two binding sites for cAMP and cGMP. The similar values of the enthalpies of denaturation obtained for both cAMP–protein and cGMP–protein complexes support this view.

The binding of cAMP and cGMP to CAP protein has been studied by Takahashi et al. (1980). The authors reported two binding sites for cAMP, with a binding constant equal to 3.9 × 10^4 M^{-1} at pH 7.0, 20 °C, and ionic strength 0.2 M, and two for cGMP, with a binding constant equal to 4.3 × 10^4 M^{-1} at pH 8.0, 20 °C, and ionic strength 0.2 M. These authors also reported an enthalpy of binding of cAMP to protein equal to −1.7 kcal mol^{-1} at 20 °C, derived from the temperature variation of the binding constant.

The values of ΔH_{obsd} (sum of all components) are 14 ± 9 and 12 ± 7 kcal mol^{-1} higher in the presence of cAMP and cGMP, respectively, than in their absence. According to Hess's law, these differences are to be attributed to the enthalpy of dissociation of the ligands, amounting to 7 and 6 kcal (mol of ligand)$^{-1}$ for cAMP and cGMP, respectively, on the basis of the assumption that two molecules of mononucleotide are bound. The dissociation enthalpy for cAMP is much larger than the value derived by Takahashi et al. (1980) from equilibrium measurements under very different conditions (lower ionic strength, 20 °C).

From the denaturation of CAP in the presence of cAMP and cGMP, the mean values of $\Delta H_{vH}/\Delta H$ are 3.1 and 2.6, respectively. This suggests that in the presence of either cAMP or cGMP what is being observed is denaturation of an aggregated species (i.e., a hexamer in the case of cAMP). As judged by the values of $\Delta H_{vH}/\Delta H$, the extent of oligomerization of CAP is increased in the presence of either mononucleotide, more so in the case of cAMP than cGMP. The aggregation of CAP at the high concentrations used in calorimetric experiments is not unexpected in view of the fact that CAP is known to bind to DNA cooperatively (Saxe & Revzin, 1979; Takahashi et al., 1979). Several authors have proposed that CAP interacts directly with or contacts RNA polymerase (Gilbert, 1976; deCrombrugghe et al., 1984) or other proteins (Hahn et al., 1984) when bound to DNA and that these contacts are responsible for stimulation of transcription by CAP.

The thermal denaturation of CAP in the presence of cAMP is qualitatively different from that in the presence of cGMP, as is apparent from inspection of Figures 2 and 3. The thermal denaturation curve for cGMP–CAP has two closely occurring components that appear as a single broad transition, while that for cAMP–CAP has three visibly apparent components. When CAP is thermally denatured in the presence of N^6-butyl-cAMP, a very broad pretransition and a main transition are observed without any evidence of the third component seen for cAMP–CAP (data not shown). These differences are consistent with the results of biochemical studies that divide these nucleotides into three different classes (Ebright et al., 1985). cGMP binds to CAP with no detectable structural change (class C), N^6-butyl-cAMP binds and causes a global alteration of structure (class D), while cAMP, in addition, stimulates DNA binding and transcription (class A). The DSC results indicate structural differences between complexes of these classes of nucleotides with CAP even in the absence of DNA.

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