Cross-Linking of the cAMP Receptor Protein of *Escherichia coli* by *o*-Phenylenedimaleimide as a Probe of Conformation†

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ABSTRACT: Reaction of the cAMP (cyclic adenosine 3′–5′-monophosphate) receptor protein (CRP) of *Escherichia coli* with the bifunctional reagent *o*-phenylenedimaleimide (oPDM) results in the cross-linking of the two subunits of a CRP protomer. In the presence of cAMP the rate of cross-linking increases. CRP modified with oPDM retains [^{3}H]cAMP binding activity but loses [^{3}H]d(I-C)$_n$ binding activity. Proteolysis of cross-linked CRP gives distinct sodium dodecyl sulfate–polyacrylamide gel electrophoretic patterns depending upon whether cAMP was present during the reaction with oPDM. CRP cross-linked in the absence of cAMP retains the same relative resistance to proteolysis as unmodified CRP. The presence of 0.1 mM cAMP during proteolysis results in the production of two fragments, one of ~13 000 daltons and a second of ~20 000 daltons. CRP cross-linked with oPDM in the presence of cAMP (then dialyzed to remove cAMP) remains sensitive to α-chymotrypsin digestion even in the absence of added cAMP producing only the 13 000-dalton fragment. It is suggested that the nature of the oPDM cross-link is a consequence of the conformational state of CRP.

The cAMP receptor protein (CRP)[1] mediates the expression of many inducible operons in *Escherichia coli* (Emmer et al., 1970; Zubay et al., 1970). cAMP elicits a conformational change in CRP (Krakow & Pastan, 1973; Wu et al., 1974) allowing it to bind to its site in the promoter region of the operon (Majors, 1975; Mitra et al., 1975), where it acts to facilitate the formation of preinitiation complexes between RNA polymerase and the promoter site (deCrombrugghe et al., 1971).

CRP consists of two identical 22 500-dalton subunits each of which contains 2 cysteinyl residues (Anderson et al., 1971). In native CRP one cysteine per subunit is readily titratable by sulfhydryl reagents (Anderson et al., 1971). Evidence suggests that the two available cysteinyl groups of the CRP protomer are located in the β region of CRP which contains the DNA-binding domain. Titration of these groups with Nbs_2 results in the loss of cAMP-dependent DNA binding activity which is regenerated by displacement of the Nbs with sulfhydryl reagents (Eilen & Krakow, 1977b). The αCRP core protein, produced by limited proteolytic digestion of the cAMP–CRP complex, lacks the two available cysteine groups, as well as the capacity to bind DNA, yet retains cAMP binding ability (Krakow & Pastan, 1973; Eilen et al., 1978).

The conformational change induced by cAMP has been shown to alter the position of the available cysteinyl residues. Fluorescent probes, covalently linked to the sulfhydryl groups, show changes in their emission spectra in the presence of cAMP, interpreted as shifts in their microenvironment (Wu et al., 1974). Eilen & Krakow (1977b) have shown that cAMP stimulates the Nbs_2 mediated formation of a disulfide bond cross-linking the two subunits of a CRP protomer. To further study the relationship between the two cysteinyl groups as a probe for conformational changes in the β region of CRP, the bifunctional reagent *o*-phenylenedimaleimide (oPDM) has been employed in the presence and absence of cAMP. Phenylenedimaleimides have been utilized to study the structure of ribosomes (Chang & Flaks, 1972), RNA polymerase (Hillel & Wu, 1977), and other multipolypeptide systems (Hasselbach & Taugner, 1970).

Materials and Methods

Materials. oPDM was obtained from Aldrich. cAMP, cGMP, 5′-AMP, Hepes, Bistris-propane, α-chymotrypsin, α-chymotrypsinogen, and crystalline ovalbumin were products of Sigma. cTuMP was generously supplied by Dr. Ira Pastan. Nbs_2 and $PhCH_2SO_2F$ were obtained from Pierce Chemical Co. Crystalline bovine serum albumin, sperm whale myoglobin, and horse heart cytochrome *c* were products of Schwarz/Mann. $NaDodSO_4$ was obtained from Bio-Rad Laboratories. [^{3}H]cAMP, [^{14}C]NEM, and Liquifluor were purchased from New England Nuclear. [^{3}H]d(I-C)$_n$ was prepared with *E. coli* DNA polymerase (Jovin et al., 1969) using [^{3}H]dCTP purchased from New England Nuclear. Nitrocellulose filters (0.45-μm pore size) were obtained from Matheson-Higgins and soaked 30 min in 0.1 M KOH before use. GFC glass fiber filters were purchased from Whatman. Ethylene glycol monomethyl ether was a product of Beckman. All reactions were carried out in polystyrene tubes.

The cAMP receptor protein and the αCRP core protein were prepared by a method presented elsewhere (Eilen et al., 1978). CRP was isolated from *E. coli* K-12 containing the KLF 41 episome as supplied by Dr. Ira Pastan.

Preparation of oPDM Cross-Linked CRP. Stock solutions of 4 mM oPDM were made in acetone. When required, dilutions were made in 0.1 M Hepes, pH 8, just prior to use. Unless otherwise stated, cross-linked samples were prepared by addition of a tenfold molar excess of oPDM to CRP in reaction mixtures containing 0.1 M Hepes, pH 8, and 0.1 mM cAMP where indicated. The CRP concentration was routinely 1 mg/mL. After incubation at 37 °C for 90 min, DTT was added to a final concentration of 40 mM. Samples of 1–2 mL were dialyzed at 4 °C for 5 h vs. 1-L of 10 mM Tris-HCl, pH 8, 1 mM EDTA, and 0.5 M NaCl and then overnight vs. 2

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[1] Abbreviations used: CRP, cAMP receptor protein; αCRP, resistant core formed by subtilisin digestion in the presence of cAMP; cAMP, adenosine 3′–5′-monophosphate; cGMP, guanosine 3′–5′-monophosphate; cTuMP, tubercidin 3′–5′-monophosphate; oPDM, *N*,*N*′-(1,2-phenylene)bismaleimide; pPDM, *N*,*N*′-(1,4-phenylene)bismaleimide; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); Nbs, thionitrobenzoate; NEM, *N*-ethylmaleimide; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; $NaDodSO_4$, sodium dodecyl sulfate; DTT, dithiothreitol; ME, β-mercaptoethanol; EDTA, (ethylenedinitrilo)tetraacetic acid; Bistris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; d(I-C)$_n$, double-stranded polydeoxynucleotide consisting of alternating dIMP and dCMP residues; MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide.

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L of 10 mM Tris-HCl, pH 8, 1 mM EDTA, and 0.1 M NaCl. Approximately 10% of the protein precipitated out of solution. Samples were therefore centrifuged at 10000 rpm for 20 min in a Beckman J-21 rotor and the supernatant protein was used. Under these conditions, 95–100% of the protein was cross-linked as determined from densitometric tracings following electrophoresis on 12% $NaDodSO_4$–polyacrylamide gels. Control samples were given the same treatment as oPDM modified CRP.

[^3H]cAMP Binding. The assay procedure was essentially that of Anderson et al. (1971). Ammonium sulfate precipitates were collected on Whatman GFC glass fiber filters, washed with 2 mL of 60% saturated ammonium sulfate, and extracted with 10 mL of a solution of 3:5 parts of ethylene glycol monomethyl ether–Liquifluor toluene and counted in a Beckman LS-230 liquid scintillation counter.

[^3H]d(I-C)$_n$ Binding. Assay mixtures contained (final volume 0.25 mL) 40 mM Bistris-propane, pH 8, 2 μg of CRP, 3.5 nmol of [^{3}H]d(I-C)$_n$ (3490 cpm per nmol), and 0.4 mM cAMP. Mixtures were incubated 5 min at 37 °C. After addition of 0.75 mL of 50 mM NaCl, the mixtures were filtered onto nitrocellulose membranes which were then dried and counted in Liquifluor toluene.

Scatchard Analysis of cAMP Binding. Scatchard analysis (Scatchard, 1949) was performed using an ultrafiltration method described by Paulus (1969). Binding assays contained (final volume 0.25 mL) 10 μg of CRP, 40 mM Bistris-propane, pH 8, and [^{3}H]cAMP (530 cpm per pmol) in concentrations ranging between 10^{-6} and 10^{-4} M. The ultrafiltration cell and PM-10 membranes were obtained from Medical Research Apparatus Corp., Boston, MA.

Protein Determinations. Protein was determined by the method of Schaffner & Weissmann (1973) using bovine serum albumin as a standard.

Polyacrylamide Gel Electrophoresis. Electrophoresis on $NaDodSO_4$–polyacrylamide gels was performed, using the method of Laemmli (1970), at 30 mA/slab gel in an appparatus purchased from the Aquabogue Machine Shop, Aquabogue, Long Island, N.Y. The gels were stained 30 min at 60 °C with 0.2% Coomassie Blue made up in the destaining solution of 25% isopropyl alcohol, 10% acetic acid. The densitometer on the Beckman Acta III spectrophotometer was used to scan strips cut from the slab gels. Protein peaks were quantitated using a Hruden planimeter.

Results

Treatment of CRP with oPDM, in the presence or absence of cAMP, leads to the cross-linking of the two CRP subunits as shown by $NaDodSO_4$–polyacrylamide gel electrophoresis. Densitometric tracings of CRP samples, treated with a tenfold excess of oPDM for 90 min at 37 °C in the absence (B) or presence (C) of 0.1 mM cAMP, show the disappearance of the 22500-dalton CRP subunit band (A) and the appearance of a 45000-dalton band corresponding to two cross-linked subunits (Figure 1). Samples of CRP cross-linked in the absence of cAMP show an additional faint band of slightly lower mobility, observed as a split band (B), the nature of which is presently not understood.

To determine whether the cross-linked subunits were derived from two CRP protomers as opposed to an intraprotomer cross-link, samples determined to be 95–100% cross-linked were applied to a Sephadex G-100 column (Figure 2). CRP cross-linked in the presence or absence of 0.1 mM cAMP eluted in the same position as unmodified CRP, indicating that the cross-link occurs within a CRP protomer and not between two protomers.

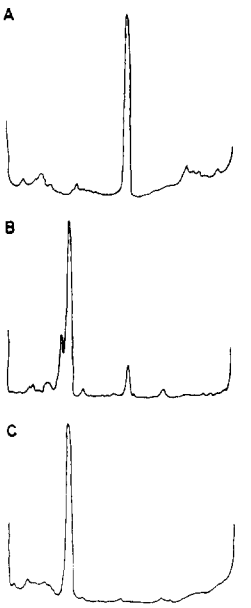

FIGURE 1: Cross-linking of CRP by oPDM. Samples containing 5 μg of unmodified CRP or CRP modified with oPDM in the presence or absence of 0.1 mM cAMP, as described under Materials and Methods, were adjusted to 0.1% $NaDodSO_4$, 10% sucrose, and 1 mM DTT in a final volume of 0.1 mL, incubated for 10 min at 60 °C, and applied in 40-μL aliquots to a 12% $NaDodSO_4$–polyacrylamide slab gel. Electrophoresis and densitometry were performed as described under Materials and Methods. Unmodified CRP (A); CRP modified with oPDM in the absence of cAMP (B); CRP modified with oPDM in the presence of cAMP (C).

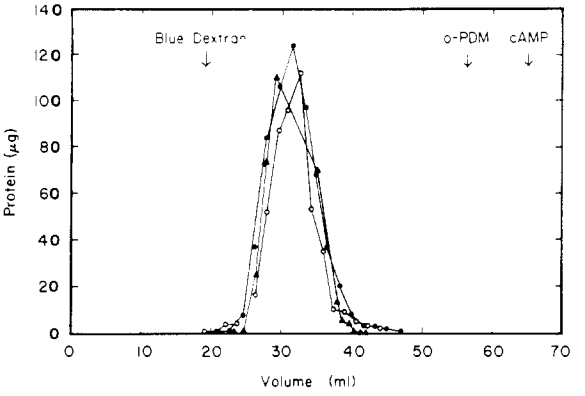

FIGURE 2: Sephadex G-100 chromatography of unmodified and oPDM cross-linked CRP. Cross-linked samples were prepared as described under Materials and Methods with the exception that samples were not dialyzed. Samples containing approximately 0.5 mg of protein in 0.5 mL were applied to a 1 × 55 cm Sephadex G-100 column equilibrated with 10 mM potassium phosphate buffer, pH 7, 1 mM EDTA, 0.1 M NaCl, and 1 mM DTT. Fractions of 1 mL were collected and protein determinations performed as described under Materials and Methods. Unmodified CRP (▲); CRP cross-linked in the absence of cAMP (○); CRP cross-linked in the presence of 0.1 mM cAMP (●).

cAMP enhances the rate of subunit cross-linking (Figure 3). The half-time ($t_{1/2}$) for the initial rate of cross-linking by a tenfold excess of oPDM over CRP at 37 °C was approximately 4 min in the absence of cAMP, while in the presence of 0.1 mM cAMP the $t_{1/2}$ was less than 1 min.

Analogues of cAMP such as cGMP or 5′-AMP, which do not produce conformational transitions required for DNA binding (Krakow, 1975), do not stimulate the rate of cross-linking by oPDM (Table I). cTuMP, an active analogue of cAMP with respect to stimulation of *gal* operon transcription (Nissley et al., 1971) and production of conformational change in CRP (Krakow, 1975), also increases the rate of cross-

Table I: Effect of Cyclic Nucleotides on the Cross-Linking of CRP by oPDM[a]

nucleotide added	concn (M)	% cross-linking
none		36
5'-AMP	10^{-3}	31
cGMP	10^{-3}	35
cAMP	10^{-5}	24
	10^{-4}	55
	10^{-3}	85
cTuMP	10^{-5}	34
	10^{-4}	83
	10^{-3}	80

[a] Reaction mixtures contained (final volume 50 μL) 5 μg of CRP (0.11 nmol), 50 mM Hepes, pH 8, 0.11 nmol of oPDM, and the indicated concentrations of nucleotides. After incubation for 10 min at 37 °C, reactions were terminated by addition of ME to a final concentration of 40 mM. The preparation and resolution of samples by $NaDodSO_4$–polyacrylamide gel electrophoresis on 10% slab gels were performed as described in Figure 3.

linking. In the experiment shown in Table I, the presence of 10^{-4} M cTuMP increased the amount of cross-linked CRP to 80% from 35%, observed in the absence of added ligands. A similar increase in the proportion of cross-linked CRP occurs in the presence of 10^{-3} M cAMP. The ability of cTuMP to effect a conformational change in CRP at concentrations approximately tenfold lower than cAMP has been previously observed (Krakow, 1975).

The functional properties of oPDM cross-linked CRP were studied (Table II). Control samples, subjected to the same treatment as modified CRP, show characteristic cAMP binding properties (Anderson et al., 1971). The enhanced ability of CRP to bind [^{3}H]d(I–C)$_n$ in the presence of cAMP is evidenced by a ninefold increase in the retention of [^{3}H]-d(I–C)$_n$ on nitrocellulose filters by the CRP–cAMP complex. CRP modified in the absence of cAMP appears to have a lower affinity for cAMP, reflected by the three- to fourfold higher dissociation constant, and may bind more than one cAMP molecule. CRP modified in the presence of cAMP shows a similar dissociation constant and number of cAMP binding sites as unmodified CRP. Inhibition of cAMP-dependent DNA binding is not as complete as that observed for samples treated in the absence of cAMP. Although differences in the nature of CRP modified with oPDM in the presence of cAMP compared with CRP modified in the absence of cAMP are suggested, the data primarily indicate that oPDM modified CRP loses DNA binding activity, while cAMP binding remains relatively unaffected.

Conditions employed in these experiments would permit the reaction of maleimides with sulfhydryl and amino groups (Brewer & Riehm, 1967). To ascertain their involvement with oPDM cross-links, the available sulfhydryls of CRP were

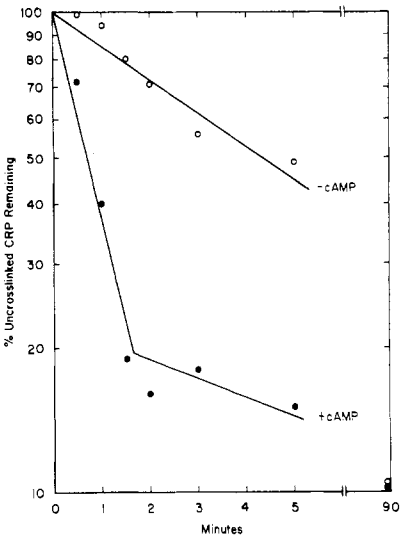

FIGURE 3: Effect of cAMP on the rate of CRP cross-linking by oPDM. The reaction mixtures contained (final volume 0.5 mL) 50 mM Hepes, pH 8, 110 μg of CRP (2.4 nmol), 25 nmol of oPDM, and 0.1 mM cAMP where indicated. The mixtures were incubated at 37 °C and, at indicated time points, 50-μL aliquots were removed and 2 μL of 1 M ME was added to terminate the reaction. The samples were adjusted to 0.1% $NaDodSO_4$ and 10% sucrose in a final volume of 0.1 mL, incubated for 10 min at 60 °C, and applied in 40-μL aliquots to a 10% $NaDodSO_4$–polyacrylamide slab gel. Electrophoresis and densitometry to determine the percent of cross-linking were performed as described under Materials and Methods. CRP cross-linked minus cAMP (○); CRP cross-linked plus cAMP (●).

titrated with the specific sulfhydryl reagent Nbs_2 (Eilen & Krakow, 1977b), prior to oPDM modification. Pretreatment of CRP with Nbs_2 completely blocks the formation of oPDM cross-links, suggesting that at least one cysteinyl residue may be involved in the cross-linking (data not shown). While Nbs_2 modification can be reversed by sulfhydryl reagents, oPDM modification is irreversible. Subsequent to oPDM modification of CRP and oPDM modification of Nbs_2 pretreated CRP, all samples were treated with mercaptoethanol to remove excess reagents, to displace Nbs linked to CRP, and to reduce any Nbs_2 mediated disulfide bonds (Eilen & Krakow, 1977b). Assay of CRP function, as described in Table II, indicates that Nbs_2 protects the loss of [^{3}H]d(I-C)$_n$ binding activity exhibited by oPDM modified samples (data not shown). The presence of Nbs linked to CRP or a disulfide bond in CRP (Eilen & Krakow, 1977b) appears to block oPDM modification, preventing the loss of DNA activity and the formation of cross-links. Monofunctional substitution by oPDM, in other regions, may account for the inability of Nbs_2 to prevent the decrease in cAMP activity resulting from oPDM treatment in the absence of cAMP. The lower affinity of these samples for cAMP does not affect their ability to bind [^{3}H]d(I-C)$_n$

Table II: Effect of oPDM on the cAMP Receptor Protein[a]

	untreated	treated, −cAMP	treated, +cAMP
% cross-linking		95	100
cAMP binding			
K_d (M)	$1.6 \pm 0.2 \times 10^{-5}$	$4.4 \pm 0.6 \times 10^{-5}$	$1.3 \pm 0.5 \times 10^{-5}$
no. of binding sites	1.1 ± 0.2	1.5 ± 0.1	1.2 ± 0.1
[^{3}H]d(I-C)$_n$ binding (nmol)			
−cAMP	0.15	0.03	0.10
+cAMP	1.27	0.05	0.33

[a] Samples were prepared as described under Materials and Methods. The cAMP binding dissociation constant and number of cAMP binding sites per CRP protomer were determined by Scatchard analysis, described under Materials and Methods. Results show the standard deviation for measurements from four sets of experimental data. DNA binding assays were performed as described under Materials and Methods.

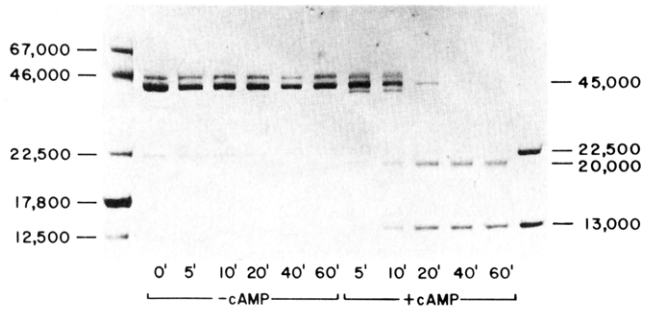

FIGURE 4: Kinetic study of α-chymotrypsin digestion of CRP cross-linked with oPDM in the absence of cAMP. Cross-linked CRP was prepared as described under Materials and Methods. Proteolysis reaction mixtures contained (final volume 0.35 mL) 50 mM Tris-HCl, pH 8, 87.5 μg or unmodified or cross-linked CRP, and 1.75 μg of α-chymotrypsin and 1 mM cAMP where indicated. Incubations were at 30 °C. At indicated time intervals, 50-μL aliquots were removed and $PhCH_2SO_2F$ was added to a final concentration of 0.1 mM. The preparation and resolution of these samples by $NaDodSO_4$–polyacrylamide gel electrophoresis on 12% slab gels were performed as described in Figure 1. The first lane shows molecular weight standards: bovine serum albumin (67 000), ovalbumin (46 000), CRP (22 500), sperm whale myoglobin (17 800), αCRP (12 500). The last lane shows unmodified CRP incubated with chymotrypsin and 1 mM cAMP for 60 min.

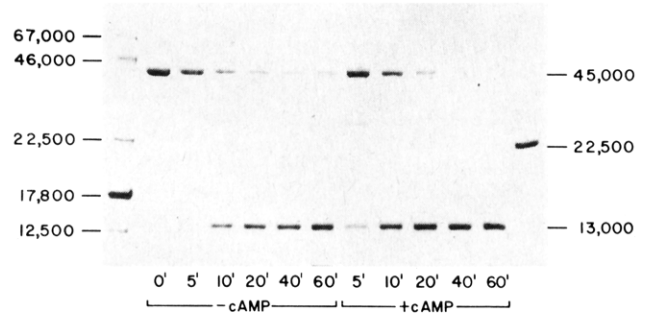

FIGURE 5: Kinetic study of α-chymotrypsin digestion of CRP cross-linked with oPDM in the presence of cAMP. The experiment was performed as described in Figure 5, except that the last lane shows unmodified CRP incubated with only chymotrypsin for 60 min.

presumably since excess cAMP is used in the assay mixtures.

While oPDM treated CRP demonstrates significant cAMP binding activity, the question remains as to whether modified CRP shows a functional response to cAMP. Proteolysis has proved a useful probe for examining cAMP induced conformational change in CRP (Krakow & Pastan, 1973; Krakow, 1975) and was therefore employed to similarly investigate oPDM cross-linked CRP.

Figures 4 and 5 show the chymotryptic digestion pattern of CRP cross-linked in the absence or presence of cAMP. Native CRP digested in the presence of cAMP is shown in the last lane of Figure 4, while CRP digested in the absence of cAMP is shown in the last lane of Figure 5. A 13 000-dalton CRP core is produced only in the presence of cAMP; in the absence of cAMP, CRP is resistant to protease. Likewise, CRP cross-linked in the absence of cAMP remains resistant to proteolysis after incubation with chymotrypsin for 60 min. The presence of 0.1 mM cAMP during proteolysis effects the production of an approximately 20 000-dalton fragment and a 13 000-dalton fragment corresponding to the CRP core. Since a 20 000-dalton fragment is not observed after digestion of unmodified CRP, it must represent a cross-linked peptide.

CRP which has been cross-linked in the presence of cAMP (Figure 5) remains susceptible to chymotrypsin digestion in the absence of cAMP; only the 13 000-dalton fragment is generated. The presence of 0.1 mM cAMP during proteolysis

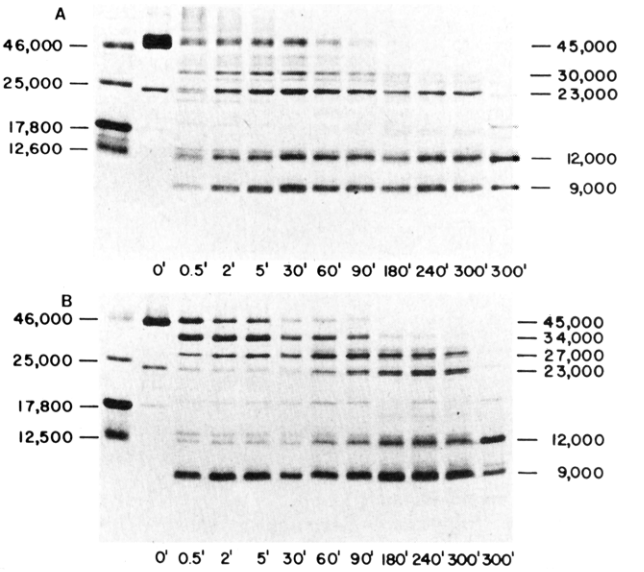

FIGURE 6: Limited proteolysis of CRP cross-linked with oPDM in the absence (A) and presence (B) of cAMP. Cross-linked CRP was prepared as described under Materials and Methods. Predigestion mixtures contained 324 μg of cross-linked or native CRP, 0.125 M Tris-HCl, pH 6.8, 1 mM EDTA, 1 mM DTT, 10% glycerol, and 0.5% $NaDodSO_4$ in a final volume of 1.2 mL. The mixtures were heated in a boiling water bath for 5 min. After cooling, 24 μg of α-chymotrypsin was added. Incubation was at 37 °C. At indicated time intervals, 20- and 100-μL aliquots were removed for protein determination and gel electrophoresis, respectively. Proteolysis was stopped in the 100-μL samples by heating in a boiling water bath for 2 min. Aliquots of 40 μL were directly applied to a 15% slab gel with a 4.2% spacer. Protein determinations and electrophoresis were performed as described under Materials and Methods. Molecular weight standards are shown in the first lane: ovalbumin (46 000), α-chymotrypsinogen (25 000), sperm whale myoglobin (17 800), horse heart cytochrome c (12 600). The last lane shows α-chymotrypsin digestion of unmodified CRP.

increases the rate of digestion but does not induce the production of additional fragments.

Similar results have been observed following subtilisin or trypsin digestion of cross-linked CRP (data not shown). In general, CRP cross-linked in the absence of cAMP shows a marked difference in sensitivity to proteolysis compared with CRP cross-linked in the presence of cAMP, suggesting that CRP may have been trapped in different conformational states.

The proteolysis experiments indicate that cAMP can induce conformational change in cross-linked CRP. Chymotrypsin digestion of CRP cross-linked in the absence of cAMP results in the production of 20 000- and 13 000-dalton fragments only when concentrations of cAMP of 10^{-5} M or higher are present during proteolysis; relatively high concentrations of cAMP increase the rate of digestion of CRP cross-linked in the presence of cAMP, although substantial digestion occurs in its absence (data not shown). cGMP cannot replace cAMP in altering the sensitivity of cross-linked CRP to proteolysis (data not shown).

The proteolysis experiments imply that the presence of cAMP during the oPDM reaction affects the nature of the cross-link. A simple and rapid method of peptide mapping by limited proteolysis in $NaDodSO_4$ (Cleveland et al., 1977) was employed to study CRP cross-linked in the presence or absence of cAMP.

In Figures 6A and 6B, distinct chymotryptic fragment patterns of cross-linked CRP are observed depending upon whether cAMP had been present during oPDM modification. At early time periods, 0.5–30 min, digests of CRP cross-linked in the absence of cAMP (Figure 6A) show prominent high

molecular weight bands of ~30 000 and 23 000; less visible are bands of 34 000–36 000 and a band of ~27000. Two smaller polypeptide fragments of 12 000 and 9000 are produced at the early time intervals. In comparison, digestion of CRP cross-linked in the presence of cAMP (Figure 6B) from 0.5 to 30 min generates three major fragments of 34 000, 27 000, and 9000; the 23 000 and 12 000 fragments are only slightly visible. As proteolysis progresses from 60 to 300 min, CRP cross-linked in the absence of cAMP breaks down into three stable fragments of ~23 000, 12 000, and 9000. Beginning at 60 min, digests of CRP cross-linked in the presence of cAMP show the gradual decrease of the 34 000 mol wt band, while two new bands of 23 000 and 12 000 appear and increase in amount with time; after 300 min, four fragments remain stable, 27 000, 23 000, 12 000, and 9000. In another experiment (data not shown), an additional 24 μg of chymotrypsin added after 180 min did not lead to further digestion of the fragments observed in Figures 6A and 6B. While fragments of similar size have been generated in digests of CRP cross-linked in the absence and presence of cAMP, the rate of appearance and stability of these fragments differ.

Chymotrypsin digestion of unmodified CRP in $NaDodSO_4$ generates two stable fragments of 9000 ± 500 and 12 000 ± 500, as observed in the last lane of Figures 6A and 6B. These fragments coincide in size with the lower molecular weight fragments appearing in digests of cross-linked CRP and it is assumed that the same segments of CRP are represented in each case. In experiments to be presented elsewhere, comparison of chymotryptic digests of native CRP and the αCRP core protein suggests that the 9000 mol wt polypeptide is derived from the amino proximal CRP core region. Since less than 20% of the native CRP protein is lost by proteolysis, the 12 000 mol wt fragment may represent at least part of the DNA binding domain of CRP.

The β region of CRP contains two available sulfhydryl groups as well as the DNA binding domain (Eilen & Krakow, 1977b; Eilen et al., 1978). Labeling these groups with a radioactive sulfhydryl reagent prior to chymotrypsin digestion of CRP would provide a means of identifying which of the fragments corresponds to the β region. Treatment of CRP with [^{14}C]NEM under native and denaturing conditions resulted in the binding of 2 and 4 mol of [^{14}C]NEM per mol of CRP, respectively. After thorough dialysis, the samples were digested with chymotrypsin in the presence of $NaDodSO_4$. Following gel electrophoresis of the digests, fragment bands were excised and the protein was eluted and counted as described in Table III. When CRP is labeled with [^{14}C]NEM under native conditions, 80% of the label is found in the 12 000 mol wt fragment and the remainder is present in the 9000 mol wt fragment, indicating that the 12 000 mol wt fragment contains the available sulfhydryl groups. When all four sulfhydryl residues are titrated with [^{14}C]NEM under denaturing conditions, the label is almost equally distributed between the 12 000 and 9000 mol wt fragments of CRP. As predicted, the 9000 mol wt fragment appears to be derived from the cAMP binding domain of CRP containing the cysteinyl residues which are only accessible to sulfhydryl reagents under denaturing conditions (Eilen & Krakow, 1977a).

In digests of CRP cross-linked in the absence (Figure 6A) and presence (Figure 6B) of cAMP, the 9000 mol wt polypeptide appears to be the only fragment produced at the same rate in both samples. If, as the evidence suggests, oPDM modification occurs within the β region of CRP, cleavage of sites within the CRP core region should not be affected. The apparently unimpeded production of the 9000 mol wt fragment from both cross-linked samples suggests that it is derived from the α-core region of CRP.

Table III: [^{14}C]NEM Modification[a]

	^{14}C (cpm)	
	native conditions	denaturing conditions
undigested CRP	1958	4458
CRP fragment mol wt		
22 500	83	110
12 000	1398	2377
9 000	346	2053

[a] Location of [^{14}C]NEM label on α-chymotryptic fragments of CRP. Reaction mixtures contained (final volume 0.5 mL) 0.5 mg of CRP, 20 mM sodium phosphate buffer, pH 8, and 0.4 mM [^{14}C]NEM (specific activity, 13 000 cpm/nmol). Reactions under denaturing conditions also contained 0.5% $NaDodSO_4$. Incubations were at 37 °C for 40 min. Reactions were terminated by addition of DTT to a final concentration of 40 mM. Samples were dialyzed overnight at room temperature vs. 2 L of 10 mM sodium phosphate, pH 7, and 0.1 M NaCl. α-Chymotryptic digestion of [^{14}C]NEM-labeled CRP was performed as described in Figure 8. Electrophoresis on 15% $NaDodSO_4$-polyacrylamide slab gels was as previously described except that slabs were minimally stained and destained to allow detection of protein bands. The radioactivity in protein bands was determined using the technique of Goodman & Matzura (1971). Bands were excised and allowed to air-dry overnight in scintillation vials. Protein was eluted by layering 0.2 mL of a 99:1 (v/v) solution of 30% H_2O_2 and concentrated ammonium hydroxide over the bands and incubating at 37 °C overnight. A 10-mL solution of ethylene glycol monomethyl ether–Liquifluor (3:5 parts) was added to the dissolved bands and the samples were counted. The counting efficiency was 30%.

It is difficult to suggest a possible origin of the 12 000 mol wt fragment which is produced at different rates from CRP cross-linked in the presence or absence of cAMP. Since the 12 000 mol wt polypeptide formed from native CRP contains the two available sulfhydryl groups, one or both of which appear to be involved in the oPDM cross-link, it is reasonable to assume that the 23 000 and 27 000 mol wt fragments represent two cross-linked peptides from the DNA binding domain. This being the case, the 12 000 mol wt fragment either must be derived from another segment of CRP or must be a product of further digestion of the larger fragments, perhaps composed of two cross-linked polypeptides.

Discussion

Two physically distinct regions have been defined in CRP; binding of cAMP to the core region elicits a conformational change which effects a functional DNA binding domain (Eilen & Krakow, 1977a,b; Eilen et al., 1978). The oPDM cross-link occurs within the β region containing the DNA binding domain and appears to involve a sulfhydryl group from at least one of the two subunits comprising a CRP protomer. While it is possible that the sulfhydryl groups in CRP which are essential for DNA binding activity are located within the DNA binding domain, it is also conceivable that modification of the sulfhydryl groups may induce a conformational change some distance away from the modified residues, thereby altering the DNA binding activity. The conformational state of CRP induced by cAMP and its active analogue cTuMP appears more favorable for the cross-linking reaction. The shift of the sulfhydryl groups to a less polar environment when cAMP binds to CRP (Wu et al., 1974) may account for an increased reactivity of the aromatic oPDM with CRP. The proximity of the residues to be bridged by oPDM, forming a 5-Å

cross-link (Chang & Flaks, 1972), would also influence the cross-linking rate. Studies of Nbs_2 modified CRP indicate that cAMP causes the sulfhydryl groups to move within close proximity of each other allowing disulfide interchange reactions, mediated by Nbs_2, which lead to the formation of an intraprotomer disulfide bond between two CRP subunits (Eilen & Krakow, 1977b). Movement of the polypeptide chains may result from an increased flexibility of the DNA binding domain upon cAMP binding to CRP. Comparative studies of oPDM and pPDM show that both compounds more rapidly cross-link CRP in the presence of cAMP (unpublished results). In view of data previously published which indicate that the sulfhydryls come within 2 Å of each other (Eilen & Krakow, 1977b), the ability of pPDM to form a 12-Å cross-link (Chang & Flaks, 1972) attests to the flexible nature of the DNA binding domain in the presence of cAMP.

In the absence of cAMP, the DNA binding domain appears to be closely associated with the core region explaining its resistance to proteolytic attack (Krakow & Pastan, 1973). cAMP may act to alter the conformation of the binding region permitting interaction with DNA and access to proteolytic enzymes. In the presence of cAMP, oPDM cross-links CRP in a manner which prevents the reassociation of the DNA binding region with the core domain after cAMP has been removed by dialysis. CRP cross-linked in this manner consequently remains susceptible to enzymatic attack regardless of whether cAMP is present during proteolysis (Figure 5). CRP cross-linked in the absence of cAMP retains its resistance to proteolysis but is not held in a "tight" conformation; upon addition of cAMP during proteolysis, the DNA binding domain polypeptides become available and digestion occurs producing the 13 000-dalton CRP core and a 20 000-dalton fragment which may represent a cross-linked peptide (Figure 4).

The results suggest the possibility that different amino acid residues are linked depending upon whether cAMP was present during oPDM modification. Experiments where CRP was pretreated with Nbs_2 prior to oPDM modification indicate a cysteinyl residue as at least one of the functional groups involved in the cross-link, assuming that the effect of Nbs_2 is not due to steric hindrance or altered charge properties of the DNA binding domain. Potentially, linkages may occur between SH + SH, SH + NH_2 or two SH + NH_2 residues. Charge differences between CRP and the αCRP core indicate that positively charged residues reside in the DNA binding domain (Eilen et al., 1978). *m*-Maleimidobenzoyl-*N*-hydroxysuccinimide (MBS) is a heterobifunctional reagent which reacts with sulfhydryl and amino groups (Kitagawa & Aikawa, 1976). We have found that MBS can cross-link CRP subunits (unpublished results) implying that amino groups are potentially within range for formation of SH–NH_2 cross-links of oPDM. Investigations comparing the nature of MBS and oPDM cross-linked CRP are currently in progress. The conjecture that different combinations of amino acid residues have reacted with the two functional groups of oPDM, depending upon whether cAMP was present during modification, is inferred by the distinct electrophoretic peptide patterns produced by digestion of cross-linked samples with chymotrypsin in the presence of $NaDodSO_4$, although remaining structural differences resulting from the effect the cross-links may have on the binding of $NaDodSO_4$ to CRP (Pitt-Rivers & Impiombato, 1968) may also explain the observed differences. $NaDodSO_4$ may not cause complete denaturation of a protein (Tanford, 1968). Thus it is also possible that the different results observed following limited proteolysis in $NaDodSO_4$ may reflect differences in the residual conformation of CRP cross-linked in the presence and absence of cAMP rather that in the amino acids which have been cross-linked. The possibility of the occurrence of monofunctional oPDM substitutions and intrapolypeptide cross-links also cannot be ruled out. Peptide mapping and amino acid analysis would clarify the extent of modification and the location of oPDM residues in CRP.

The present study has shown oPDM to be a useful probe for investigating the conformational structures of CRP. In particular, the cAMP-induced DNA binding state appears to be "frozen" by the oPDM cross-link. Limited proteolysis of native and cross-linked CRP with chymotrypsin, in the presence of $NaDodSO_4$, has produced electrophoretic patterns from which fragments have been identified that are likely to contain segments of the DNA binding domain. The results also indicate the feasibility of the separation and isolation of the DNA binding domain by selective cleavage under appropriate conditions. Such an approach has been successfully utilized in the isolation of the DNA binding "headpieces" of the *lac* repressor protein (Geisler & Weber, 1977).

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References

Anderson, W. B., Schneider, A. B., Emmer, M., Perlman, R. L., & Pastan, I. (1971) *J. Biol. Chem. 246*, 5929–5937.
Brewer, C. F., & Riehm, J. P. (1967) *Anal. Biochem. 18*, 248–255.
Chang, F. N., & Flaks, J. G. (1972) *J. Mol. Biol. 68*, 177–180.
Cleveland, D. W., Fischer, S. G., Kirschner, M. W., & Laemmli, U. K. (1977) *J. Biol. Chem. 252*, 1102–1106.
deCrombrugghe, B., Chen, B., Anderson, W. B., Nissley, P., Gottesman, M., Pastan, I., & Perlman, R. (1971) *Nature (London), New Biol. 231*, 139–142.
Eilen, E., & Krakow, J. S. (1977a) *Biochim. Biophys. Acta 493*, 115–121.
Eilen, E., & Krakow, J. S. (1977b) *J. Mol. Biol. 114*, 47–60.
Eilen, E., Pampeno, C., & Krakow, J. S. (1978) *Biochemistry 17*, 2469–2473.
Emmer, M., deCrombrugghe, B., Pastan, I., & Perlman, R. L. (1970) *Proc. Natl. Acad. Sci. U.S.A. 66*, 480–487.
Geisler, N., & Weber, K. (1977) *Biochemistry 16*, 938–843.
Goodman, D., & Matzura, H. (1971) *Anal. Biochem. 42*, 481–486.
Hasselbach, W., & Taugner, G. (1970) *Biochem. J. 119*, 265–271.
Hillel, Z., & Wu, C.-W. (1977) *Biochemistry 16*, 3334–3342.
Jovin, T. M., Englund, P. T., & Bertsch, L. L. (1969) *J. Biol. Chem. 244*, 2996–3008.
Kitagawa, T., & Aikawa, T. (1976) *J. Biochem. (Tokyo) 79*, 233–236.
Krakow, J. S. (1975) *Biochim. Biophys. Acta 383*, 345–350.
Krakow, J. S., & Pastan, I. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2529–2533.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Majors, J. (1975) *Nature (London) 256*, 672–674.
Mitra, S., Zubay, G., & Landy, A. (1975) *Biochem. Biophys. Res. Commun. 67*, 857–863.
Nissley, P., Anderson, W. B., Gottesman, M. E., Perlman, R. L., & Pastan, I. (1971) *J. Biol. Chem. 246*, 4671–4678.
Paulus, H. (1969) *Anal. Biochem. 32*, 91–100.

Pitt-Rivers, R., & Impiombato, F. S. A. (1968) *Biochem. J. 109*, 825–829.
Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660–672.
Schaffner, W., & Weissmann, G. (1973) *Anal. Biochem. 56*, 502–514.
Tanford, C. (1968) *Adv. Protein Chem. 23*, 211–217.
Wu, F. Y.-H., Nath, K., & Wu, C.-W. (1974) *Biochemistry 13*, 2567–2572.
Zubay, G., Schwartz, D., & Beckwith, J. (1970) *Proc. Natl. Acad. Sci. U.S.A. 66*, 104–110.

Electron-Transferring Enzymes in the Plasma Membrane of the Ehrlich Ascites Tumor Cell†

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ABSTRACT: The plasma membrane of the Ehrlich ascites tumor cell contains an NADH dehydrogenase. This activity was shown not to be due to contamination by other subcellular membranes. A variety of electron acceptors have been compared as to rate with the following result: ferricyanide ≫ cytochrome *c* > cytochrome b_5 > glyoxylate > dichlorophenolindophenol. Oxygen acceptance could not be detected. The optimum assay temperature and pH ranges were 30–40 °C and pH 6–8, respectively. With respect to either NADH or ferricyanide, the kinetics yielded linear double-reciprocal plots. Inhibition of the enzyme by sulfhydryl reagents could be blocked by excess NADH. Detergents such as Triton X-100 or cholate resulted in solubilization of the enzymatic activity, but phospholipase A_2 did not. The activity differed from that of the mitochondria in that it was not inhibited by rotenone or antimycin A. The possible involvement of NADH oxidation in the energetics of plasma membrane transport is discussed.

In bacteria the plasma membrane is well-known to be the location of electron transport. In contrast, the corresponding functions of eukaryotic cells have been assumed to be localized in the endoplasmic reticulum and mitochondrial membranes. Only recently has attention returned to possible electron transport in the plasma membranes of animal cells. Although either NADH or NADPH oxidoreductase activities have been shown in almost every isolated plasma membrane preparation tested, they have usually been considered to be due to contamination by other subcellular membranes. Recent evidence indicates, however, that one or both of these enzymes may be intrinsic to the plasma membrane. This topic has been reviewed by Löw & Crane (1978).

Recently, several investigations have indicated that neither cellular ATP nor alkali-ion gradients can fully explain energization of amino acid transport across plasma membranes (Christensen et al., 1973; Schafer et al., 1977; Banay-Schwartz et al., 1974). We have considered the possibility that the plasma membrane NADH oxidation may play a role in energizing membrane transport. Inhibition of amino acid uptake by the flavin antagonist quinacrine and restoration of transport in energy-depleted cells by pyruvate or phenazine methosulfate indicated that perhaps a mitochondrial energy source other than ATP may drive plasma membrane transport (Garcia-Sancho et al., 1977). The implications of this proposal have led us to an investigation of the NADH dehydrogenase in membrane vesicles from the Ehrlich cell. The present report provides evidence that the NADH oxidizing activity is due to a plasma-membrane-bound enzyme and demonstrates some of its characteristics.

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Experimental Procedures

Isolation of Plasma Membrane Vesicles. Ehrlich ascites tumor cells were maintained by intraperitoneal injection of Swiss mice and harvested between 8 and 10 days after transfer. The cells were filtered through cheesecloth during collection in an ice-cold beaker. All procedures were performed at 4 °C unless specified otherwise in the text. The cells were washed free of red blood cells in 0.9% saline by centrifuging at 900*g* for 30 s. This step was followed by two washings in 15 mM Tris, pH 7.5, 15 mM NaCl, and 1 mM $MgCl_2$ (buffer A) with centrifugation at 2000*g* for 3 min. After suspension in an equal volume of buffer A, the cells were kept on ice for a minimum of 20 min. The swollen cells were then homogenized in a glass Dounce homogenizing apparatus fitted with a tight pestle. Complete cellular disruption was ensured by checking the homogenate with a light microscope. The homogenate was centrifuged at 650*g* for 10 min and the cloudy supernatant was discarded. From here on, the procedure differs from that used earlier (Im et al., 1976). The pellet was resuspended in buffer A with a glass rod and centrifuged at 500*g* for 5 min. The resulting pellet was taken up in buffer A and dispersed with two to three gentle strokes in the Dounce homogenizer. The homogenate was then diluted and centrifuged for 5 min at 60*g*, followed by 3 min at 500*g*. The crude plasma membrane fraction floats as a snow-white layer over a pinkish tan pellet. The supernatant may contain significant amounts of plasma membrane if homogenization was too vigorous. The white layer was carefully removed, diluted with buffer A, and washed free of any residual nuclei by centrifuging at 60*g* for 5 min. The supernatant was then centrifuged at 12000*g* for 10 min. The resulting pellet was mixed with 94.3% (w/v) sucrose in a ratio of 3:7 (10 mL of total volume) and overlayered in turn with 10 mL of 54.1% (w/v) sucrose, 10 mL of 48.5% (w/v) sucrose, and 8 mL of 39.0% (w/v) sucrose. These gradients were centrifuged for 2 h at 100000*g*. The layer of membranes at the 39.0–48.5% interface was collected

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