Photoaffinity Labeling of the Adenosine Cyclic 3′,5′-Monophosphate Receptor Protein of *Escherichia coli* with 8-Azidoadenosine 3′,5′-Monophosphate†

Hiroji Aiba and Joseph S. Krakow*

ABSTRACT: Photoaffinity labeling of the cAMP receptor protein (CRP) of *Escherichia coli* with 8-azidoadenosine 3′,5′-monophosphate (8-N_3cAMP) has been demonstrated. 8-N_3cAMP is able to support the binding of (^{3}H)d(I-C)$_n$ by CRP, indicating that it is a functional cAMP analogue. Following irradiation at 254 nm, (^{32}P)-8-N_3cAMP is photocross-linked to CRP. Photolabeling of CRP by (^{32}P)-8-N_3cAMP is inhibited by cAMP but not by 5′AMP. The data indicate that (^{32}P)-8-N_3cAMP is covalently incorporated following binding at the cAMP binding site of CRP. The (^{32}P)-8-N_3cAMP–CRP digested with chymotrypsin was analyzed by NaDodSO$_4$–polyacrylamide gel electrophoresis. Of the incorporated label, one-third remains associated with the amino-proximal α core region of CRP [Eilen, E., Pampeno, C., & Krakow, J. S. (1978) *Biochemistry 17*, 2469] which contains the cAMP binding domain; the remaining two-thirds of the label associated with the β region are digested. Limited proteolysis of the (^{32}P)-8-N_3cAMP–CRP by chymotrypsin in the presence of NaDodSO$_4$ shows the radioactivity to be distributed between the molecular weight 9500 (amino-proximal) and 13 000 (carboxyl-proximal) fragments produced. These results suggest that a part of the carboxyl-proximal region is folded over and close enough to the cAMP binding site to be cross-linked by the photoactivated (^{32}P)-8-N_3cAMP bound at the cAMP binding site.

The cyclic AMP receptor protein (CRP)[1] in the presence of cAMP facilitates the transcription of catabolite repressible operons by binding to a site within the promoters of these operons (Musso et al., 1977; Majors, 1975; Mitra et al., 1975; Lee et al., 1974; Pastan & Adhya, 1976). Binding of cAMP by CRP results in a conformational change involved in DNA binding (Krakow & Pastan, 1973; Wu et al., 1974; Eilen & Krakow, 1977a,b). CRP contains a single cAMP binding site although CRP consists of two apparently identical 22 500 molecular weight subunits (Anderson et al., 1971). A functional differentiation within the CRP protomer has been demonstrated. Proteolytic digestion of CRP in the presence of cAMP yields a resistant core. The αCRP core resulting from subtilisin digestion is composed of two ~12 500 molecular weight polypeptides (Krakow & Pastan, 1973; Eilen et al., 1978) representing the amino-proximal segment of the CRP subunit (Schlesinger, 1978). The CRP cores produced after digestion by subtilisin, chymotrypsin, trypsin, or the *Staphylococcus aureus* V8 protease bind cAMP but have lost DNA binding activity (Pampeno & Krakow, 1978). All or part of the cAMP binding domain of CRP is located within the amino-proximal segment of the CRP subunit.

Affinity labeling provides a direct approach toward defining the binding sites of CRP. In this paper we describe the photoaffinity labeling of CRP with 8-azidoadenosine 3′,5′-monophosphate (Haley, 1977), a functional analogue of cAMP. Azide compounds photolyze readily to produce a highly reactive nitrene derivative capable of reaction with proteins (Bayley & Knowles, 1977). 8-N_3cAMP has been used as a photoaffinity label to modify cAMP binding proteins in several eucaryotic systems (Haley, 1975; Pomerantz et al., 1975; Walter et al., 1977).

Materials and Methods

Materials. CRP was purified by the procedure of Eilen et al. (1978) from *Escherichia coli* K-12 containing the KLF 41 episome. (^{32}P)-8-N_3cAMP (32–57 Ci/mmol) was purchased from ICN, and (^{3}H)cAMP (37 Ci/mmol), (^{3}H)dCTP (23 Ci/mmol), and Liquifluor were purchased from New England Nuclear. cAMP, 8-N_3cAMP, cGMP, 5′AMP, dITP, and α-chymotrypsin were products of Sigma Chemical Co. The *E. coli* DNA polymerase I was obtained from Miles Laboratories. (^{3}H)d(I-C)$_n$ (1600 cpm/nmol) was synthesized according to Jovin et al. (1969) using *E. coli* DNA polymerase I, (^{3}H)dCTP, and dITP. Nitrocellulose filters (0.45-μm pore size) were obtained from Matheson Higgens, and EGF glass-fiber filters were obtained from Enzo Biochem. Inc. All other reagents were of the best grade available.

Photoaffinity Labeling Assay. All experiments were performed in small Pyrex test tubes (1.1-cm diameter × 10 cm). Typical reaction mixtures contained (final volume 100 μL) 0.8–18 μg of CRP, 0.2–5.0 μM (^{32}P)-8-N_3cAMP (2000–500 cpm/pmol), 10–250 μM 5′AMP or cAMP, and 10 mM potassium phosphate, pH 7.6 (at 4 °C). The mixtures were incubated at 37 °C for 30 min and then irradiated with ultraviolet light from a 15-W low-pressure mercury lamp (General Electric). The lamp was placed just above the top of the test tubes containing the samples. After irradiation 100 μL of 10^{-2} M ATP was added to each reaction mixture and incubated for 5 min at 37 °C, followed by addition of 200 μL of cold 20% Cl_3CCOOH. The Cl_3CCOOH precipitate was collected on an EGF filter and washed with cold 5% Cl_3CCOOH, and the dried filter was counted in 5 mL of Liquifluor–toluene.

† From the Department of Biological Sciences, Hunter College of the City University of New York, New York, New York 10021. *Received August 3, 1979; revised manuscript received January 7, 1980.* This work was supported by a research grant from the National Institutes of Health (GM 22619).

[1] Abbreviations used: CRP, cyclic AMP receptor protein; 8-N_3cAMP, 8-azidoadenosine 3′,5′-monophosphate; cAMP, adenosine 3′,5′-monophosphate; cGMP, guanosine 3′,5′-monophosphate; NaDodSO$_4$, sodium dodecyl sulfate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; Cl_3CCOOH, trichloroacetic acid.

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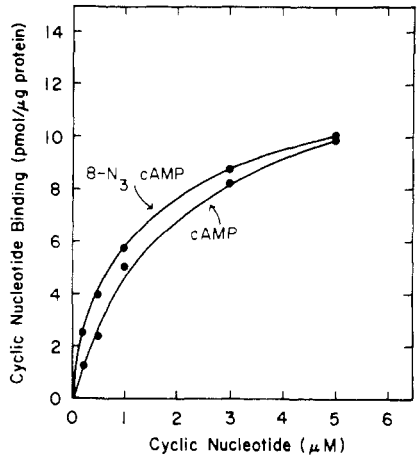

FIGURE 1: Reversible binding of (^{32}P)-8-N_3cAMP by CRP. The solutions contained the following in 100 μL: 10 mM potassium phosphate, pH 7.6, 10 mM 5'AMP, 2.5 μg of CRP, and (^{3}H)cAMP or (^{32}P)-8-N_3cAMP at the indicated concentration.

For time course experiments, 0.5-mL reaction mixtures were irradiated; 100 μL of the mixture was removed at different intervals of irradiation and assayed as described above.

The irradiation dose was estimated according to Johns (1968) using 1,3-dimethyluridine. At the distance of 10 cm, the dose rate was 13.6 nEinsteins/(cm^2 min).

Protease Digestion of Labeled CRP. A reaction mixture of 5 mL containing 225 μg of CRP, 10^{-6} M (^{32}P)-8-N_3cAMP, 5×10^{-5} M 5'AMP, and 10 mM potassium phosphate, pH 7.6, was incubated at 37 °C for 30 min and irradiated for 10 min at a dose rate of 10 nEinsteins/(cm^2 min). The irradiated mixture was dialyzed against three changes of 1 L of 10 mM Tris-HCl (pH 7.6), 0.1 M NaCl, 1 mM EDTA, and 1 mM DTT and concentrated by vacuum dialysis using a Micro-ProDiCon (Biomolecular Dynamics) at 4 °C. The concentrated sample was subjected to protease digestion in the absence and presence of $NaDodSO_4$ as described in the figure legends.

Electrophoresis of the digested samples was performed on $NaDodSO_4$–polyacrylamide (15%) slab gels by the method of Laemmli (1970), using an apparatus manufactured by the Aquabogue Machine Shop, Aquabogue, NY. The gels were stained for 30 min at 60 °C with 0.1% Coomassie blue. The optical density profile at 580 nm was measured for each gel channel. Protein was quantitated by weighing the peak areas cut from the recorder paper. To determine the radioactivity profile, the gel strips were cut into 0.2- or 1-cm segments, dried, and directly counted in 5 mL of Liquifluor–toluene.

Binding Assay. Assays for the binding of (^{3}H)cAMP and (^{32}P)-8-N_3cAMP were carried out as previously described (Krakow & Pastan, 1973), Ammonium sulfate precipitates were collected on EGF glass fiber filters, extracted with 10 mL of a solution of 3:5 ethylene glycol monomethyl ether–Liquifluor–toluene and counted.

Binding of (^{3}H)d(I-C)$_n$ was assayed by a modified method of Krakow & Pastan (1973). Reaction mixtures containing (final volume 250 μL) 2.5 μg of CRP, 4.5 nmol of (^{3}H)d(I-C)$_n$, 8-N_3cAMP, or cAMP at the indicated concentration and 50 mM Tris-HCl, pH 8 (at 4 °C), were incubated for 5 min at 37 °C. After addition of 0.75 mL of 50 mM NaCl, the mixtures were filtered onto nitrocellulose membranes presoaked for 30 min in 0.1 M KOH. The dried filters were counted in 5 mL of Liquifluor–toluene.

Other Methods. Protein was determined by the method of Schaffner & Weissmann (1973), using bovine serum albumin as a standard. Radioactivity was measured by a Beckman LS-230 liquid scintillation counter, while the optical density was measured by a Beckman Acta III spectrophotometer.

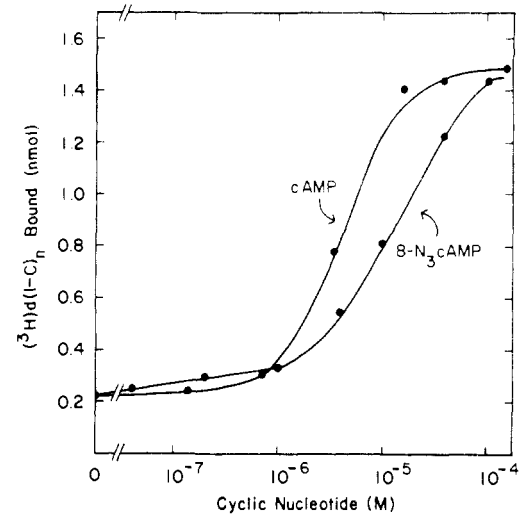

FIGURE 2: Effect of 8-N_3cAMP on binding of (^{3}H)d(I-C)$_n$ by CRP. The solutions contained the following in 250 μL: 50 mM Tris-HCl, pH 8.0, 4.5 nmol of (^{3}H)d(I-C)$_n$ (1600 cpm/nmol), 2.5 μg of CRP, and 8-N_3cAMP or cAMP at the indicated concentration.

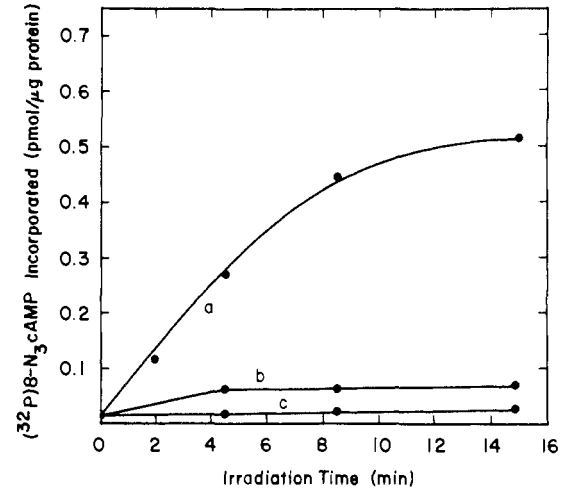

FIGURE 3: Time course for photoaffinity labeling of CRP. The reaction mixtures contained the following in 0.5 mL: 10 mM potassium phosphate, pH 7.6, 1 μM (^{32}P)-8-N_3cAMP plus 50 μM cAMP or 50 μM 5'AMP, and 22.5 μg of CRP or bovine serum albumin. The solutions were incubated for 30 min at 37 °C and then irradiated under the standard conditions [37 °C and 13.6 nEinsteins/(cm^2 min)]. At the times indicated, 100-μL aliquots were assayed for Cl_3CCOOH-precipitable radioactivity. (a) CRP plus 5'AMP; (b) CRP plus cAMP; (c) bovine serum albumin plus 5'AMP.

Results

8-N_3cAMP Is a Functional Analogue of cAMP. Figure 1 shows that the reversible binding of (^{32}P)-8-N_3cAMP to CRP is very similar to that of cAMP; the dissociation constants were estimated to be 1.1×10^{-6} M and 1.8×10^{-6} M, respectively, for 8-N_3cAMP and cAMP. The data indicate that 8-N_3cAMP and cAMP bind to the same site on CRP.

8-N_3cAMP was able to stimulate binding of (^{3}H)d(I-C)$_n$ by CRP (Figure 2). The concentrations of 8-N_3cAMP and cAMP required for half-maximal stimulation were approximately 1×10^{-5} and 4×10^{-6} M, respectively. Although 8-N_3cAMP was slightly less effective than cAMP, the results indicate that the analogue binds to the cAMP binding site,

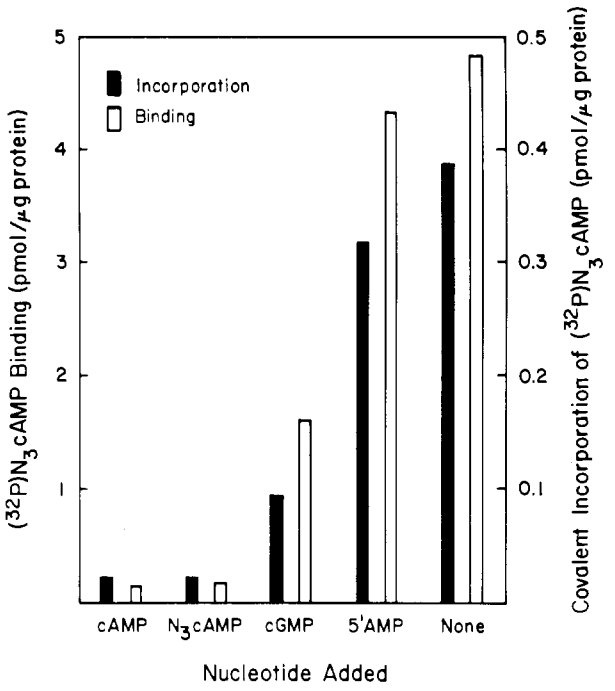

FIGURE 4: Effect of nucleotides on photolabeling and reversible binding. The solutions contained the following in 150 μL: 10 mM potassium phosphate, pH 7.6, 6.8 μg of CRP, 1 μM (^{32}P)-8-N_3cAMP, and 0.1 mM of the indicated nucleotide. The solutions were incubated for 30 min at 37 °C, and a 50-μL aliquot was removed to assay for binding. The remaining 100 μL was irradiated for 15 min at 37 °C and assayed for Cl_3CCOOH-precipitable radioactivity.

eliciting the conformational change in CRP requisite to DNA binding.

Characterization of Photoaffinity Labeling. Figure 3 shows the time course of photoaffinity labeling of CRP by (^{32}P)-8-N_3cAMP following irradiation at 254 nm. In the presence of 10^{-6} M (^{32}P)-8-N_3cAMP and 5×10^{-5} M 5'AMP (Figure 3a), Cl_3CCOOH-precipitable radioactivity increased with time, approaching a plateau after 15 min of irradiation. The Cl_3CCOOH-precipitable radioactivity cross-linked to CRP was not affected by heating the sample (previously irradiated for 15 min) for 5 min at 100 °C prior to acid precipitation. At 15-min irradiation (corresponding to a dose of 200 nEinsteins/cm^2) the photo-cross-linked yield was 0.025 mol of (^{32}P)-8-N_3cAMP linked per mol of CRP. Photoactivated incorporation was markedly inhibited by 5×10^{-5} M cAMP (Figure 3b). Photolysis of (^{32}P)-8-N_3cAMP prior to incubation with CRP resulted in no detectable incorporation. Cl_3CCOOH-precipitable incorporation of (^{32}P)-8-N_3cAMP was insignificant when bovine serum albumin replaced CRP (Figure 3c). The results indicate that 8-N_3cAMP can be used to specifically react at the cAMP binding site of CRP.

Figure 4 shows the results of experiments in which the reversible binding and the photoactivated incorporation of (^{32}P)-8-N_3cAMP were measured in the presence of cAMP analogues. It is apparent that the competition patterns for binding and incorporation are parallel, further supporting the specificity of the photoactivated incorporation of 8-N_3cAMP. If the photoactivated incorporation requires the specific binding of 8-N_3cAMP, the incorporation yield should decline with decreasing binding activity of CRP. To check this point, we partially inactivated CRP by heating at 65 °C for various times. Binding and photoactivated incorporation of (^{32}P)-8-N_3cAMP to partially inactivated CRP were determined. The results are summarized in Table I. It is seen that there is a close relation between binding activity and the incorporation

Table I: Effect of Heat Inactivation of CRP on Photoaffinity Labeling[a]

incubn at 65 °C (min)	reversible binding pmol/μg of CRP	reversible binding %	photoactivated incorpn pmol/μg of CRP	photoactivated incorpn %
0	8.84	100	0.401	100
1	5.48	62	0.243	61
3	4.09	46	0.175	44
10	1.82	21	0.071	18

[a] Solutions initially contained the following in 100 μL: 20 mM potassium phosphate, pH 7.6, and 9 μg of CRP (200 pmol). After being heated at 65 °C for the times indicated, (^{32}P)-8-N_3cAMP and 5'AMP were added to give a concentration of 1 and 50 μM, respectively. After 30 min at 37 °C an aliquot of 50 μL was removed for assay of binding. The remaining 150 μL was irradiated at 254 nm for 15 min at 37 °C and then assayed for Cl_3CCOOH-precipitable radioactivity.

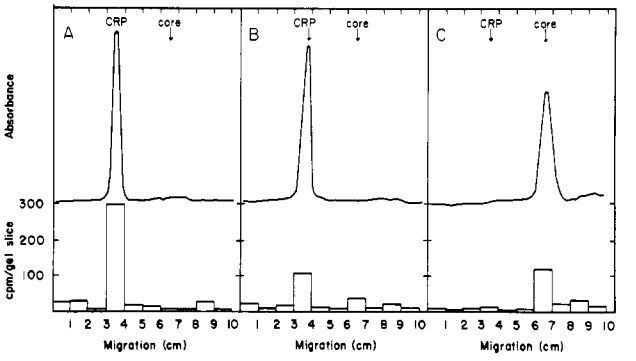

FIGURE 5: Chymotrypsin digestion of photolabeled CRP. Digestion mixtures contained the following in 50 μL: 5 μg of photolabeled CRP, 10 mM Tris-HCl, pH 7.6, 0.1 M NaCl, 1 mM EDTA, 1 mM DTT, 0.5 μg of chymotrypsin (B and C), and 1 mM cAMP (C). Mixtures were incubated at 37 °C for 30 min, and the reaction was terminated by the addition of 2 μL of 2.5 mM $PhCH_2SO_2F$. The samples were brought to 0.1% $NaDodSO_4$ and 10% glycerol (final volume 50 μL) and heated for 10 min at 60 °C and then layered on a $NaDodSO_4$–polyacrylamide slab gel, followed by electrophoresis. Densitometric tracings and the counting of radioactivity in 1-cm gel slices were carried out as described under Materials and Methods. (A) No digestion; (B) digestion in the absence of cAMP; (C) digestion in the presence of cAMP.

yield. All experiments strongly indicate that the photoactivated incorporation of (^{32}P)-8-N_3cAMP into CRP occurs at the cAMP binding site.

Proteolytic Cleavage of Photo-Cross-Linked CRP. Since the data indicated that photolabeling of CRP by 8-N_3cAMP occurred at the cAMP binding site, it was of interest to define where in CRP the modification occurred. Limited proteolysis provides a convenient procedure for characterizing the region modified. Proteolytic digestion of CRP in the presence of cAMP yields a core which retains cAMP binding activity, while CRP in the absence of cAMP is relatively resistant to proteolysis (Krakow & Pastan, 1973; Eilen et al., 1978). CRP modified by photolabeling with (^{32}P)-8-N_3cAMP was dialyzed to remove noncovalently bound nucleotides and concentrated. Approximately 1% of the CRP in this preparation was cross-linked to the photoactivated (^{32}P)-8-N_3cAMP. The cAMP binding activity of the irradiated CRP was comparable to that of nonirradiated CRP, indicating that irradiation did not in itself significantly damage CRP.

$NaDodSO_4$–polyacrylamide gel electrophoresis showed that the incorporated radioactivity comigrated with the 22 500 molecular weight CRP subunit (Figure 5A). Incubation of the photolabeled preparation with chymotrypsin in the absence

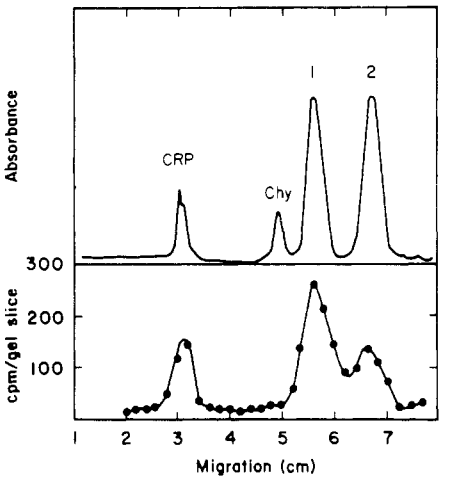

FIGURE 6: Chymotrypsin cleavage in $NaDodSO_4$ of photolabeled CRP. The mixture containing (final volume 100 μL) 10 μg of photolabeled CRP, 0.125 M Tris (pH 6.8), 1 mM EDTA, 1 mM DTT, 10% glycerol, and 0.5% $NaDodSO_4$ was heated in a boiling water bath for 2 min. After being cooled, 1.5 μg of chymotrypsin was added, and the reaction mixture was incubated for 30 min at 37 °C. The digestion was terminated by heating the mixture for 2 min in boiling water. The entire sample was then subjected to $NaDodSO_4$–polyacrylamide (15%) slab gel electrophoresis. The densitometric tracing and the counting of radioactivity of each gel slice (0.2 cm) were carried out as described under Materials and Methods.

of cAMP did not result in appreciable digestion of CRP; however, only one-third of the radioactivity originally present was recovered in the 22 500 molecular weight polypeptide (Figure 5B). An additional minor radioactive region was present in the region corresponding to the 13 000 molecular weight core. When the chymotryptic digestion was carried out in the presence of cAMP, all of the 22 500 molecular weight polypeptide was degraded and replaced by the ~13 000 molecular weight core fragment. Approximately one-third of the radioactivity cross-linked to CRP was recovered in the core (Figure 5C). The results suggest that while the cAMP binding site is located in the core region cross-linking following photoactivation of (^{32}P)-8-N_3cAMP was largely directed to a closely associated segment of the carboxyl-proximal region of the CRP polypeptide.

In the presence of $NaDodSO_4$, chymotrypsin cleaves CRP into two fragments of molecular weights ~13 000 and ~9500. The 9500 molecular weight fragment is derived from the amino-proximal region of CRP and is presumed to contain at least part of the cAMP binding domain (Pampeno & Krakow, 1978). Approximately 90% of the mixture of irradiated CRP plus photolabeled CRP was cleaved by chymotrypsin in the presence of $NaDodSO_4$ (Figure 6). Almost all of the radioactivity present prior to digestion was accounted for in the residual uncleaved CRP plus the two fragments. The ratio of radioactivity cross-linked to the 13 000 molecular weight fragment (808 cpm) relative to that to the 9500 molecular weight fragment (421 cpm) was 2:1.

Discussion

The results presented indicate that 8-N_3cAMP served as a functional analogue of cAMP. 8-N_3cAMP stimulated (^3H)d(I-C)$_n$ binding at concentrations similar to those at which cAMP acts. In addition, the dissociation constants for reversible binding of cAMP and 8-N_3cAMP were comparable. The presence of the azide group in the 8 position of the analogue did not result in an inactive derivative, and presumably this was also true following photoactivation to form the reactive 8-nitrene cAMP derivative. The results indicate that cross-linking of photoactivated 8-N_3cAMP to CRP occurred during association with the cAMP binding site of CRP. The conditions chosen to ensure specific labeling resulted in cross-linking to only 1–3% of the CRP. Increasing either the concentration of CRP or that of (^{32}P)-8-N_3cAMP resulted in an increase in nonspecific cross-linking (not competed by the presence of cAMP during irradiation).

Our initial premise was that labeling should occur predominantly within the amino-proximal region of the CRP polypeptide. Previous results derived from digestion of CRP plus cAMP by several proteolytic enzymes showed that the resulting cores retained cAMP binding activity (Krakow & Pastan, 1973; Eilen et al., 1978). The αCRP core resulting from subtilisin digestion comprises the amino-terminal region of CRP (Schlesinger, 1978). The data presented in Figure 5 showed that most of the incorporated radioactivity derived from cross-linking by (^{32}P)-8-N_3cAMP was not recovered in the core following chymotrypsin digestion. Cleavage of labeled CRP by chymotrypsin in $NaDodSO_4$ showed a preferential cross-linking to the 13 000 molecular weight fragment. The 9500 molecular weight fragment represents the amino-proximal region present in both CRP and the CRP cores (C. Pampeno and J. S. Krakow, unpublished results). Since both CRP and the CRP cores are able to bind cAMP, the results derived from the photolabeled CRP were unexpected. Since 8-N_3cAMP is a functional cAMP analogue, it may be inferred that the 8 position of the purine moiety of cAMP is not directly involved in interaction with the functional groups present in the cAMP binding site of CRP. Thus, the photoactivated nitrene group derived from 8-N_3cAMP may react with amino acid side chains present in a region of the CRP polypeptide folded over and proximal to the cAMP binding site. Irradiation of the SAP–CRP core (subunit molecular weight 18 500) in the presence of 8-N_3cAMP resulted in covalent modification which was less than 10% of that seen with CRP, although reversible binding of 8-N_3cAMP by SAP–CRP was comparable to that observed with CRP (results not shown). The SAP–CRP core lacks about 40 amino acids present at the carboxyl-terminal region of CRP. Binding of an effective cyclic nucleotide results in a conformational change in CRP (Krakow & Pastan, 1973; Wu et al., 1974; Eilen & Krakow, 1977a,b). The carboxyl-terminal region absent in SAP–CRP may be important in maintaining the CRP conformation established in the absence of cAMP. CRP is relatively resistant to proteolysis in the absence of cAMP; the SAP–CRP core is sensitive to chymotrypsin, trypsin, or subtilisin even when cAMP is not present (Pampeno & Krakow, 1978). We would suggest that a carboxyl-terminal region may interact with and perhaps comprise part of the cAMP binding site. This would be compatible with the labeling pattern observed following covalent modification by 8-N_3cAMP.

Acknowledgments

We are grateful to Dr. Ira Pastan for supplying the *E. coli* cells carrying the KLF 41 episome. We thank Gopalan Nair for his excellent technical assistance in preparing the CRP preparation used in this study.

References

Anderson, W. B., Schneider, A. B., Emmer, M., Perlman, R. L., & Pastan, I. (1971) *J. Biol. Chem. 246*, 5927–5937.

Bayley, H., & Knowles, J. R. (1977) *Methods Enzymol. 46*, 69–114.

Eilen, E., & Krakow, J. S. (1977a) *Biochim. Biophys. Acta 413*, 115–121.

Eilen, E., & Krakow, J. S. (1977b) *J. Mol. Biol. 114*, 47–67.

Eilen, E., Pampeno, C., & Krakow, J. S. (1978) *Biochemistry 17*, 2469–2473.
Haley, B. E. (1975) *Biochemistry 14*, 3852–3857.
Haley, B. E. (1977) *Methods Enzymol. 46*, 339–346.
Johns, H. E. (1968) *Photochem. Photobiol. 8*, 547–563.
Jovin, T. M., Englund, P. I., & Bertsch, L. L. (1969) *J. Biol. Chem. 244*, 2996–3008.
Krakow, J. S., & Pastan, I. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2529–2533.
Lee, N., Wilcox, G., Gielow, W., Arnold, J., Cleary, P., & Englesberg, E. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 104–110.
Majors, J. (1975) *Nature (London) 256*, 672–674.
Mitra, S., Zubay, G., & Landry, A. (1975) *Biochem. Biophys. Res. Commun. 67*, 857–863.
Musso, R. E., DiLauro, R., Adhya, S., & deCrombrugghe, B. (1977) *Cell 12*, 847–854.
Pampeno, C., & Krakow, J. S. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 1830.
Pastan, I., & Adhya, S. (1976) *Bacteriol. Rev. 40*, 527–551.
Pomerantz, A. H., Rudolph, S. A., Haley, B. E., & Greengard, P. (1975) *Biochemistry 14*, 3858–3862.
Schaffner, W., & Weissmann, C. (1973) *Anal. Biochem. 56*, 502–514.
Schlesinger, D. H. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 36*, 1619.
Walter, U., Uno, I., Liu, A. Y.-C., & Greengard, P. (1977) *J. Biol. Chem. 252*, 6494–6500.
Wu, F.-H., Nath, K., & Wu, C.-W. (1974) *Biochemistry 13*, 2567–2572.

Aldehyde-Induced Adenosine Triphosphatase Activities of Fructose 6-Phosphate and Fructose Kinases†

Ronald E. Viola‡ and W. W. Cleland*

ABSTRACT: Chitose-6-P (2,5-anhydromannose-6-P) induces ATPase activity of fructose-6-P kinase with a V_{max} 2–3% that of the normal kinase reaction with fructose-6-P or 2,5-anhydromannitol. Chitose (and presumably also chitose-6-P) is 52% hydrated in water while chitose deuterated at C-1 is 60% hydrated because of the equilibrium isotope effect of 0.73 on aldehyde hydration. Deuterated chitose-6-P gave a normal isotope effect on V/K of 1.23, but no effect on V_{max}, showing that the free aldehyde is the activator and the hydrated form does not bind appreciably. With fructokinase, chitose can act either as a substrate, being phosphorylated at C-6 when adsorbed with C-6 next to MgATP, or as an inducer of ATPase activity when adsorbed with C-1 next to MgATP. The ATPase has a rate about 25% that of the kinase.

Kinases are enzymes which transfer the γ-phosphate of MgATP to various acceptors. While considerable information about the stereochemistry (Knowles, 1980) and the coordination of Mg to the phosphates of ATP and the products during the reaction (Cleland & Mildvan, 1979) is now available, the chemical mechanism is still not well understood. The ATPase activity seen in the presence of aldehydes corresponding to alcohols which are substrates for certain kinases is thus of considerable mechanistic interest. Such activity was first seen with glycerokinase, which was found by Janson & Cleland (1974) to phosphorylate L-glyceraldehyde with a V_{max} 150% that of glycerol but in the presence of D-glyceraldehyde to show ATPase activity with a V_{max} 30% that of glycerol. We have now found that both fructose-6-P kinase and fructokinase show ATPase activity in the presence of aldehyde analogues of their substrates.

When such activity was first observed, it was thought to result from phosphorylation of the hydrated form of the aldehyde to produce an unstable compound which decomposed nonenzymatically. This hypothesis is readily testable by measuring the deuterium isotope effects on the activation parameters of the aldehyde, since there is a 37% deuterium isotope effect on the hydration equilibrium (Hill & Milosevich, 1976; Lewis & Wolfenden, 1977). For example, an aldehyde that is half-hydrated in water will contain 58% hydrate and 42% free aldehyde when deuterated at C-1. The isotope effect on V/K will either be inverse ($^{D}(V/K) = 0.86$)[1] if the hydrate is the activator or normal ($^{D}(V/K) = 1.16$) if the free aldehyde is the activator. If only the active form can combine with the enzyme, no isotope effect on V_{max} is predicted ($^{D}V = 1.0$). However, an apparent isotope effect on V_{max} will be seen if both the aldehyde and its hydrate combine with the enzyme, but only one is active, because the ratio of the two forms will be different for deuterated and unlabeled aldehyde and the presence of a competitive inhibitor in constant ratio to the variable substrate reduces V_{max} (but has no effect on V/K). As a second example, when an aldehyde is nearly fully hydrated (96% in the case of glyceraldehyde (Swenson & Barker, 1971)), the isotope effect on V/K will either be near unity ($^{D}(V/K) = 0.99$) if the hydrate is the activator or nearly fully expressed ($^{D}(V/K) = 1.35$) if the aldehyde is the activator.

Bar-Tana & Cleland (1974) reported that 2,5-anhydromannitol is a substrate for fructose-6-P kinase and is phosphorylated slightly faster than fructose-6-P. We now report that its aldehyde analogue, 2,5-anhydromannose-6-P (chitose-6-P), induces ATPase activity by fructose-6-P kinase. Through the use of the deuterium isotope effect on aldehyde hydration, we will show that the free aldehyde of chitose-6-P

† From the Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706. *Received September 20, 1979.* Supported by grants to W.W.C. from the National Institutes of Health (GM-18938) and the National Science Foundation (BMS-16134). A preliminary report on this work has been presented (Viola, 1979).

‡ Present address: Department of Chemistry, Southern Illinois University, Edwardsville, IL 62026.

[1] ^{D}X is the deuterium isotope effect on X (that is, the ratio of X values for unlabeled and labeled molecules).

0006-2960/80/0419-1861$01.00/0