

- Hjerten, S. (1962) *Arch. Biochem. Biophys.*, **Suppl. 1**, 147.  
 Jue, R., Lambert, J. M., Pierce, L. R., & Traut, R. R. (1978) *Biochemistry* **17**, 5399-5406.  
 Laemmli, U. K. (1970) *Nature (London)* **227**, 680-685.  
 Morrissey, J. H. (1981) *Anal. Biochem.* **117**, 307-310.  
 Perham, R. N., Appella, E., DuBois, G. C., & Inman, J. K. (1980) in *Methods of Peptide and Protein Sequence Analysis* (Birrr, C., Ed.) pp 345-348, Elsevier/North-Holland, Amsterdam.  
 Peters, R., & Richards, F. M. (1977) *Annu. Rev. Biochem.* **46**, 525-551.  
 Reed, L. J. (1974) *Acc. Chem. Res.* **7**, 40-46.  
 Shapiro, A. L., Vinuela, E., & Maizel, J. V. (1967) *Biochem. Biophys. Res. Commun.* **28**, 815-820.  
 Sun, T.-T., Bollen, A., Kahan, L., & Traut, R. R. (1974) *Biochemistry* **13**, 2334-2340.  
 Thomas, J. O. (1978) in *Techniques in Protein and Enzyme Biochemistry* (Kornberg, H. L., Metcalfe, J. C., Northcote, D. H., Pogson, C. I., & Tipton, K. F., Eds.) Vol. B106, pp 1-22, Elsevier/North-Holland, Amsterdam.  
 Thomas, J. O., & Kornberg, R. D. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 2626-2630.  
 Wang, K., & Richards, F. M. (1974) *J. Biol. Chem.* **249**, 8005-8018.

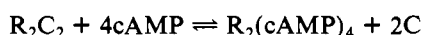
## Adenosine 3',5'-Cyclic Monophosphate Protein Kinase from Bovine Brain: Inactivation of the Catalytic Subunit and Holoenzyme by 7-Chloro-4-nitro-2,1,3-benzoxadiazole<sup>†</sup>

F. Thomas Hartl and Robert Roskoski, Jr.\*

**ABSTRACT:** NBD-Cl (7-chloro-4-nitro-2,1,3-benzoxadiazole) inactivates the catalytic subunit of adenosine 3',5'-cyclic monophosphate (cAMP) dependent protein kinase isolated from bovine brain by covalent modification. The reaction follows pseudo-first-order kinetics. The pseudo-first-order rate constant ( $k_{app}$ ) shows a hyperbolic dependence on NBD-Cl concentration, suggesting formation of a reversible complex before covalent modification with a dissociation constant ( $K_i$ ) of 150  $\mu$ M. MgATP, MgADP, and adenosine protect against inactivation 75, 60, and 50%, respectively, whereas ATP, ADP, or Mg<sup>2+</sup> alone does not protect. This process was competitive in nature ( $K_a$  for adenosine 44  $\mu$ M). Protein substrates (histone IIa, 2 mg/mL; Leu-Arg-Arg-Ala-Ser-Leu-Gly, 1 mM) do not protect against inactivation; histone actually accelerates the rate of inactivation. Inactivation is associated with modification of  $2.1 \pm 0.15$  mol of cysteine/mol of catalytic subunit, determined spectrophotometrically and radioisotopically. Activity can be restored by treating inactivated enzyme with 2-mercaptoethanol. MgATP protects one cysteine on the

average from modification while protecting against inactivation. In the absence of cAMP, type I and II regulatory subunits from bovine skeletal muscle and type II regulatory subunit from bovine brain slow modification of the catalytic subunit by NBD-Cl. In the presence of 0.6 mM cAMP the brain holoenzyme is inactivated slower than the free catalytic subunit, but type I and II holoenzymes from skeletal muscle are inactivated faster than the free catalytic subunit. This suggests that the regulatory and catalytic subunits communicate even in the presence of saturating concentrations of cAMP. The effect of brain regulatory subunit on the reactivity of sulfhydryls in the catalytic subunit is opposite that of the skeletal muscle regulatory subunits. The latter's effect on sulfhydryl reactivity is similar to that observed by Armstrong and Kaiser for the bovine heart enzyme [Armstrong, R. N., & Kaiser, E. T. (1978) *Biochemistry* **17**, 2840], suggesting differences in the interactions of the regulatory and catalytic subunits in the enzymes from these different tissues.

Adenosine 3',5'-cyclic monophosphate (cAMP) dependent protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) catalyzes the phosphorylation of polypeptidic serine and threonine residues. The enzyme consists of dissimilar regulatory (R) and catalytic (C) subunits.<sup>1</sup> Until recently the enzyme was reported to be activated by binding 2 mol of cAMP and dissociating into free C subunits and dimeric R<sub>2</sub>(cAMP)<sub>2</sub> complexes (Gill & Garen, 1970; Tao et al., 1970; Rosen & Erlichman, 1975; Beavo et al., 1975). Recent studies, however, indicate that each regulatory dimer can bind 4 mol of cAMP (Corbin et al., 1978; Weber et al., 1979), yielding the following activation scheme:



Work in this and other laboratories is aimed at identifying amino acids present at the active site of the C subunit as well as studying the mechanism of inactivation of the C subunit by regulatory proteins.

Possible amino acid residues important to enzyme function have been assessed by chemical modification studies. Characterization of ethoxyformic anhydride inhibition of C subunit (Witt & Roskoski, 1975a), for example, suggests that a tyrosine residue occurs in the active site. Chemical modification of cysteine residues also inhibits enzyme activity (Sugden et al., 1976; Peters et al., 1977; Armstrong & Kaiser, 1978).

<sup>†</sup> From the Department of Biochemistry, Louisiana State University Medical Center, New Orleans, Louisiana 70112. Received January 14, 1981; revised manuscript received April 14, 1982. This work was supported by Grant NS-15994 from the U.S. Public Health Service.

<sup>1</sup> Abbreviations: R, regulatory subunit of cAMP-dependent protein kinase; C, catalytic subunit of cAMP-dependent protein kinase; FSBA, 5'-[p-(fluorosulfonyl)benzoyl]adenosine; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); Mops, 3-(N-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Ser-peptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly; DEAE, diethylaminoethyl; NaDodSO<sub>4</sub>, sodium dodecyl sulfate.

Recently, the affinity label 5'-[*p*-(fluorosulfonyl)benzoyl]-adenosine (FSBA) was shown to inactivate the C subunit (Hixson & Krebs, 1979) by modifying a single lysine residue (Zoller & Taylor, 1979).

7-Chloro-4-nitro-2,1,3-benzoxadiazole (NBD-Cl) has been used to modify sulfhydryl, tyrosyl, and amino groups in several ATP-utilizing enzymes including mitochondrial ATPase (Ferguson et al., 1975a,b) and ( $\text{Na}^+/\text{K}^+$ )ATPase (Cantley et al., 1978). NBD-Cl reacts with these amino acid residues to form adducts with distinctive spectral and fluorescent properties (Ghosh & Whitehouse, 1968; Birkett et al., 1970; Ferguson et al., 1975a,b). These permit the identification and quantitation of the modified residues.

In this paper we report that NBD-Cl reacts with the C subunit of bovine brain cAMP-dependent protein kinase by modifying two cysteine residues. The modification of one is not inhibitory; modification of both, however, abolishes the phosphotransferase activity. Type II R subunit from brain slows the inactivation by NBD-Cl in the absence or presence of saturating concentrations of cAMP. This effect on sulfhydryl reactivity is different from that seen with type I and II R subunits from bovine skeletal muscle. It also differs from that observed for the bovine heart enzyme by Armstrong & Kaiser (1978), suggesting that the type II R subunits from bovine brain and muscle interact differently with the C subunit. Preliminary reports of these results have appeared (Hartl, 1980; Hartl & Roskoski, 1979).

#### Materials and Methods

The synthetic heptapeptide used as substrate (Leu-Arg-Arg-Ala-Ser-Leu-Gly) was purchased from Sigma or Peninsula Laboratories. Budget-Solve and [ $^{14}\text{C}$ ]NBD-Cl were obtained from Research Products International. Carrier-free  $\text{H}_3^{32}\text{PO}_4$  and [ $\gamma\text{-}^{32}\text{P}$ ]ATP were purchased from ICN. Whatman 3-MM filter paper and P81 phosphocellulose paper were obtained from the manufacturer. Epoxy-activated Sepharose 6B was purchased from Pharmacia. Other chemicals were obtained from Sigma. The method of Muneyama et al. (1971) was used to synthesize 8-[(2-hydroxyethyl)thio]adenosine 3',5'-cyclic monophosphate.

**Enzyme Preparation.** All operations were carried out at 4 °C unless otherwise specified. Bovine brain (6 kg) was homogenized for 2 min at high speed in 2 volumes of 5 mM EDTA (pH 7.0) in a blender. Phenylmethanesulfonyl fluoride (in 2-propanol) was added immediately before homogenization to give a final concentration of 40  $\mu\text{M}$ . The homogenate was centrifuged for 30 min at 13700g. The supernatant was filtered through glass wool and stirred with 3 L of DEAE-cellulose (Whatman DE-52) equilibrated with buffer A [10 mM potassium phosphate/1 mM EDTA/0.1 mM dithiothreitol (pH 6.8)] for 1 h. The resin was allowed to settle and most of the supernatant was decanted. The resin was then collected in a large scintered glass funnel and washed with 15 L of 25 mM NaCl in buffer A and then poured in a column (8.5  $\times$  60 cm) and washed with this buffer until the  $A_{280}$  of the effluent fell below 0.05 (about 25 L was required). The enzyme was eluted with a 7.5-L linear gradient of 25–500 mM NaCl in buffer A. The fractions containing cAMP-dependent protein kinase activity, which eluted at 0.2 M NaCl, were pooled and passed over a 5 mL (1.5  $\times$  2.5 cm) column of cAMP-Sepharose 6B [prepared by coupling of 8-[(2-hydroxyethyl)thio]adenosine 3',5'-cyclic monophosphate to epoxy-activated Sepharose as described by Weber et al. (1979)]. The resin was washed with 25 mL of buffer B [25 mM Mops/150 mM NaCl/1 mM EDTA/15 mM 2-mercaptoethanol (pH 6.8)]. The filtrate was saved for the

purification of the catalytic subunit (described below). The cAMP-Sepharose was washed with 200 mL of buffer B and 200 mL of 2 M NaCl in the same buffer. The resin was washed with 30 mL of 1 mM 5'-AMP and 25 mL of 1 mM cAMP. The column was washed exhaustively with buffer B to remove cAMP and eluted in one of the two following ways. (1) cAMP elution. Two column volumes of 50 mM cAMP in buffer B were passed rapidly over the column. The flow was stopped, and the column was incubated at 4 °C for 24–48 h. The R subunit was eluted by slow passage of 20 mL of 50 mM cAMP. The flow was stopped for an additional 24–48 h, and the elution procedure was repeated. A small amount of R subunit remained bound to the column; this can be eluted with 6 M urea in buffer B. (2) Urea elution. Instead of being eluted with 50 mM cAMP, the R subunit can also be eluted from the column by slow passage of 4–5 column volumes of 6 M urea in buffer B. The eluate was immediately dialyzed against buffer B (100 volumes, three changes) to remove excess cAMP or urea. Because the type II R collected from the cAMP-Sepharose generally was contaminated with several smaller molecular weight proteins that are presumably proteolytic fragments of type II R (because they bind cAMP), the eluate from the cAMP-Sepharose was concentrated to a small volume (2 mL) and chromatographed on Sephadex G-100 superfine (2.5  $\times$  70 cm) to resolve these proteins from R.

**Purification of the Catalytic Subunit.** The supernatant from the cAMP-Sepharose treatment contained C subunit activity that was 90–95% cAMP independent. This solution was dialyzed overnight against buffer A and then stirred with 500 mL of packed DEAE-cellulose (equilibrated with buffer A) for 2 h. The resin was allowed to settle, collected on a Buchner funnel, and washed with 1 volume of 50 mM potassium phosphate/0.1 mM dithiothreitol (pH 6.8). The filtrate was loaded on a 5  $\times$  6 cm column of hydroxylapatite (Bio-Gel HTP) equilibrated with this buffer. The column was washed with 2 column volumes of 100 mM potassium phosphate/0.1 mM dithiothreitol (pH 6.8) and eluted with a 1-L linear gradient of 0.1–0.4 M potassium phosphate (pH 6.8) containing this thiol. The active fractions were pooled, diluted with 2 volumes of cold distilled  $\text{H}_2\text{O}$ , and loaded on a 2  $\times$  2.5 cm column of hydroxylapatite (equilibrated as described above). The C subunit was eluted with 30 mL of 0.4 M potassium phosphate/0.1 M dithiothreitol (1-mL fractions were collected). The active fractions were pooled, concentrated to 3–5 mL, and chromatographed on a 2.5  $\times$  70 cm Sephadex G-100 superfine column [equilibrated with 50 mM sodium phosphate/100 mM NaCl/0.5 mM EDTA (pH 7.0)]. This procedure gave 6 mg of homogeneous C subunit as judged by NaDodSO<sub>4</sub>-polyacrylamide gel electrophoresis.

**Enzyme Assays.** Catalytic subunit activity was measured by the phosphocellulose paper adsorption method of Witt & Roskoski (1975b), with histone IIa or Ser-peptide as substrate. Incubations included 50 mM Mops (pH 7.0), 10 mM  $\text{MgCl}_2$ , 0.1 mM [ $\gamma\text{-}^{32}\text{P}$ ]ATP, and 5 mg/mL histone IIa. With Ser-peptide (0.15 mM) as substrate, the procedure was modified as follows: After 25- $\mu\text{L}$  portions of the reaction mixture were transferred to 1  $\times$  2 cm phosphocellulose strips, the strips were immersed in 75 mM phosphoric acid (5 mL of 85%  $\text{H}_3\text{PO}_4/\text{L}$ , pH 1.8, about 10 mL/sample). The strips were swirled for 2 min and washed twice more (2 min each) with gentle agitation. After the strips were dried at 100 °C for 5 min, the radioactivity was measured by liquid scintillation spectrometry with Budget-Solve as the scintillant. After 250 000 cpm was applied to the paper, backgrounds were 250 cpm or less. That

Table I: Purification of the Catalytic Subunit from Bovine Brain<sup>a</sup>

step	vol (mL)	protein (mg/mL)	activity (units/mL)	sp act. (units/mg)	total units	yield (%)
whole homogenate	11000	45	76.5	1.71	841 500	[100]
13700g supernatant	7110	13.2	52.8	4.0	375 400	44.6 (100)
DEAE-cellulose eluate	2000	5.0	91.8	18.4	183 600	21.8 (48.9)
post cAMP-agarose	2300	4.3	79.0	18.4	181 700	21.5 (48.4)
second DEAE-cellulose	3550	0.038	29.9	786.3	106 100	12.6 (28.3)
hydroxylapatite I	350	0.070	332	4742	116 130	13.8 (30.9)
hydroxylapatite II	18	0.63	6204	9848	111 672	13.3 (29.7)
Sephadex G-100-40	7	0.83	15373	18522	107 611	12.8 (28.7)

<sup>a</sup> One unit is defined as the amount of enzyme that will catalyze the transfer of 1 nmol of <sup>32</sup>P to Ser-peptide/min at pH 7.0 and 30 °C.

amount is less than that obtained with the 30% acetic acid wash of Glass et al. (1978) and requires less time. With a fourth 2-min wash, backgrounds of less than 100 cpm were obtained.

**Reaction of NBD-Cl with the C Subunit.** Catalytic subunit (0.01–1 mg/mL) was incubated with NBD-Cl at 30 °C in 0.2 M Mops/0.1 mM EDTA (pH 7.0). The NBD-Cl was added as a freshly prepared stock solution in acetonitrile. Because NBD-Cl is light sensitive, stock solutions and reaction mixes were protected from light. The final concentration of acetonitrile in the reaction mixtures was 1–5%; these concentrations failed to significantly alter C subunit activity. Portions were removed throughout the course of the reaction and assayed for C subunit activity as described.

**Difference Spectrophotometry.** Difference spectra of C subunit modified with NBD-Cl were recorded on a Cary Model 210 spectrophotometer. The reactions between NBD-Cl and the C subunit or *N*-acetylcysteine were set up in split-cell cuvettes (path length per half-cell 0.48 cm) at 23 °C in 0.1 M Mops buffer, pH 7.0. The cuvettes contained C subunit or *N*-acetylcysteine in one compartment and 0.4 mM NBD-Cl in the other. The cuvette in the sample beam was inverted repeatedly to mix the contents of the two chambers. After 60 min, scans were made from high to low wavelengths at 20 nm/min. The extinction coefficient for *S*-NBD-*N*-acetylcysteine of 13 000 M<sup>-1</sup> cm<sup>-1</sup> (Birkett et al., 1970) was used to determine the stoichiometry. First derivatives of the difference spectra were calculated on a Hewlett-Packard 9815S by using a program supplied by SLM Instruments, Inc.

**Determination of [<sup>14</sup>C]NBD Incorporation into the C Subunit.** C subunit (0.5–1.0 mg/mL) was incubated with 0.24 mM NBD-Cl under the conditions described for unlabeled NBD-Cl. Two methods were used to determine [<sup>14</sup>C]NBD incorporation. Method A: At various times portions were removed from the reaction mixture and precipitated with 10% trichloroacetic acid. The precipitate was collected by centrifugation at 13600g. The pellet was washed with 5% trichloroacetic acid and dissolved in 1 N NaOH, and the radioactivity and protein concentration were measured. Method B: Portions were removed from the reaction mixture and spotted on Whatman 3-MM filter paper strips, and the strips were immersed immediately in ice-cold 10% trichloroacetic acid (10 mL/strip). The strips were washed twice with 5% acid (10 mL/strip), with acetone/ethanol (1:1 v/v, 10 mL/strip), and then with petroleum ether (5 mL/strip). After an air drying, the radioactivity was measured as described below. Methods A and B yielded similar results. In subsequent experiments, we found that the incorporation of labeled NBD into C can also be measured by application to phosphocellulose strips followed by phosphoric acid washes (vide supra).

**Radioactivity and Protein Determination.** Protein concentrations were measured by the method of Lowry et al. (1951), with bovine serum albumin as the standard. Radio-

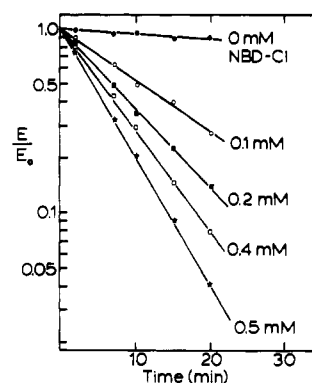


FIGURE 1: Time course of inactivation of the catalytic subunit of bovine brain cAMP-dependent protein kinase by NBD-Cl. The catalytic subunit (10 µg/mL) was incubated with the specified concentrations of NBD-Cl as described under Materials and Methods. Portions were removed at the specified times for phosphotransferase activity measurement.

activity was measured by means of liquid scintillation spectrometry with Budget-Solve as the scintillant. Counting efficiency was 71% for method A and 60% for method B.

## Results

**Purification of the Catalytic and Regulatory Subunits.** Catalytic subunit recoveries during the purification are given in Table I. About 6 mg of C subunit is routinely obtained from 6 kg of brain. Attempts to improve the yield by extracting the first precipitate with Triton X-100 were successful; however, this adversely effected the purity of the final product and was therefore not routinely used. The modest increase in activity following the first hydroxylapatite column may be related to removal of a protein kinase inhibitor.

Following elution from the cAMP affinity resin with 50 mM cAMP or 6 M urea, about 10.5 mg of regulatory subunit is obtained from 6 kg of starting material. The R subunit prepared by either procedure inhibits the C subunit at a ratio of 1 mol of R/mol of C and binds equivalent amounts of cAMP. Furthermore, the R subunit prepared by either procedure behaved similarly in the experiments with NBD-Cl.

**Inactivation of the Catalytic Subunit by NBD-Cl.** NBD-Cl inactivates catalytic subunit activity purified from bovine brain in a time-dependent fashion. After a 30-min incubation (30 °C, pH 7) with 0.5 mM NBD-Cl, for example, less than 5% of the original activity remains. If the natural logarithm of residual activity ( $E/E_0$ , where  $E$  is the activity at any time and  $E_0$  is the activity at zero time) is plotted vs. time of incubation with NBD-Cl (Figure 1), a straight line is obtained with a slope equal to an apparent pseudo-first-order rate constant ( $k_{app}$ ). This constant shows a hyperbolic dependence on NBD-Cl concentration (Figure 2). When  $1/k_{app}$  is plotted vs.  $1/[NBD-Cl]$  (inset, Figure 2), a straight line is obtained with a nonzero y intercept. This behavior is consistent with

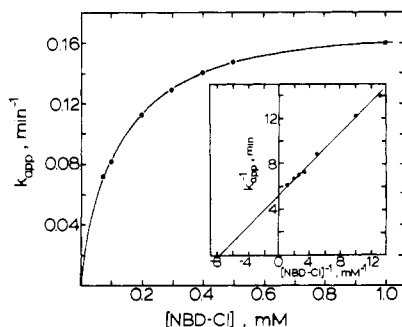


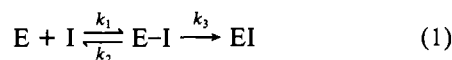
FIGURE 2: Dependence of pseudo-first-order rate constant ( $k_{app}$ ) of inactivation of the catalytic subunit on NBD-Cl concentration. The catalytic subunit (20  $\mu\text{g/mL}$ ) was incubated with various concentrations of NBD-Cl as described under Materials and Methods. The value of  $k_{app}$  was determined at each NBD-Cl concentration from the slope of the  $\ln(E/E_0)$  vs. time plot. (Inset) Double-reciprocal plot of the data.

Table II: Effect of Adenine Nucleotides and Peptide Substrates on Inactivation of the Catalytic Subunit by NBD-Cl<sup>a</sup>

additions to reaction mixture	% residual activity	
	no $\text{MgCl}_2$	10 mM $\text{MgCl}_2$
none	20	17
ATP (1 mM)	22	83
ADP (1 mM)	17	66
AMP (1 mM)	19	28
adenosine (1 mM)	59	56
histone IIa (2 mg/mL)	4	4
Ser-peptide (1 mM)	11	10
guanethidine (1 mM)	13	15

<sup>a</sup> The catalytic subunit (60  $\mu\text{g/mL}$ ) was incubated with 0.5 mM NBD-Cl in 0.2 M Mops/0.1 mM EDTA (pH 7.0) containing 2% (v/v) acetonitrile at 24 °C for 15 min. Portions were then removed for phosphotransferase activity determinations. In the absence of NBD-Cl greater than 95% of the original activity remained after 15 min; all data are corrected for this inactivation. Activity of 100% (in the absence of inhibitors) was  $1.75 \pm 0.12$   $\mu\text{mol of } ^{32}\text{P}/(\text{min} \cdot \text{mg})$  with histone IIa as the substrate.

the idea of formation of a reversible complex before covalent modification according to the scheme:



where E is the free enzyme, I is NBD-Cl, E-I is a reversible enzyme-NBD-Cl complex, and EI is the inactive covalently modified enzyme. Steady-state treatment of this process yields the equation (Kitz & Wilson, 1962):

$$\frac{1}{k_{app}} = \frac{K_i}{k_3} \frac{1}{[\text{I}]} + \frac{1}{k_3} \quad (2)$$

where  $k_{app}$  is the apparent pseudo-first-order rate constant described above and  $K_i$  is the dissociation constant of the reversible enzyme-NBD-Cl complex ( $K_i = k_2/k_1$ ). A plot of  $1/k_{app}$  vs.  $1/[\text{I}]$  yields a straight line with the ordinate intercept equal to  $1/k_3$  and the abscissa intercept equal to  $-1/K_i$ . The data in Figure 2 yield a  $K_i$  for NBD-Cl of 150  $\mu\text{M}$ .

If NBD-Cl is acting at or near the active site, substrates and inhibitors that bind at the active site should slow the rate of inactivation by NBD-Cl. Table II shows that adenine nucleotides, but not protein substrates, protect against inactivation by NBD-Cl. In the absence of  $\text{Mg}^{2+}$ , ATP and ADP (1 mM) do not protect against inactivation, but in the presence of 5 mM  $\text{Mg}^{2+}$ , ATP and ADP (1 mM) protect 75 and 60%, respectively. Surprisingly, 5'-AMP (1 mM) protects very poorly against inactivation, while adenosine (1 mM) protects 50% in the absence or presence of  $\text{Mg}^{2+}$ . The  $K_i$  values de-

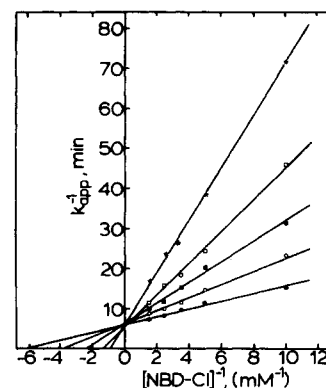


FIGURE 3: Protection of the catalytic subunit by adenosine from inactivation by NBD-Cl. The catalytic subunit (20  $\mu\text{g/mL}$ ) was incubated with varying concentrations of NBD-Cl as described under Materials and Methods in the presence of various concentrations of adenosine. Concentrations of adenosine: none ( $\bullet$ ), 25 ( $\circ$ ), 50 ( $\blacksquare$ ), 100 ( $\square$ ), and 250 ( $\star$ )  $\mu\text{M}$ .

termined by steady-state inhibition of phosphotransferase activity (data not shown) for ADP and adenosine are  $13 \pm 3$   $\mu\text{M}$  and  $42 \pm 3$   $\mu\text{M}$ , respectively. 5'-AMP, which does not protect as well as ADP or adenosine against inactivation, has a  $K_i$  of  $745 \pm 10$   $\mu\text{M}$ . Thus it appears that the greater protection from NBD-Cl-mediated inactivation afforded by ADP and adenosine at 1 mM concentrations is due to their higher affinity for the C subunit.

If NBD-Cl is acting at the active site, competitive inhibitors of C should competitively slow the rate of reaction of NBD-Cl with the C subunit. A substrate or competitive inhibitor (A) that binds at the active site with a characteristic dissociation constant ( $K_A$ ), when included in the scheme depicted in eq 1, adds the following equation:



where A competes with I for free E.

The rate equation obtained by a steady-state treatment of this process according to Kitz & Wilson (1962) follows:

$$\frac{1}{k_{app}} = \frac{K_i}{k_3} \left( 1 + \frac{[\text{A}]}{K_A} \right) \frac{1}{[\text{I}]} + \frac{1}{k_3} \quad (4)$$

where A and  $K_A$  are as described. To test the notion that NBD-Cl acts at or near the active site, we incubated C subunit with varying concentrations of NBD-Cl in the presence of various fixed concentrations of adenosine. Values of  $k_{app}$  were determined and plotted vs.  $1/[\text{NBD-Cl}]$  according to eq 4. Adenosine competitively slows the rate of inactivation of the C subunit by NBD-Cl (Figure 3). From the data plotted in accordance to eq 4, the  $K_A$  for adenosine is 44  $\mu\text{M}$ , which closely agrees with the value of 42  $\mu\text{M}$  calculated from steady-state inhibition of phosphotransferase activity (results not shown). These results are consistent with the contention that NBD-Cl acts at or near the active site of the catalytic subunit, thereby inactivating it. Alternatively, there may be a ligand-induced conformational change that inhibits inactivation by NBD-Cl.

**Identification of the Modified Residues.** NBD derivatives of amino acids have distinctive absorption spectra (Birkett et al., 1970; Ferguson et al., 1975a; Aboderin et al., 1973) that allow the modified residues to be identified. To determine the identity and number of the amino acids modified, we incubated C subunit with 0.5 mM NBD-Cl at pH 7.0 in the sample beam of a spectrophotometer. The resulting difference spectrum is shown in Figure 4. The broad positive absorption band

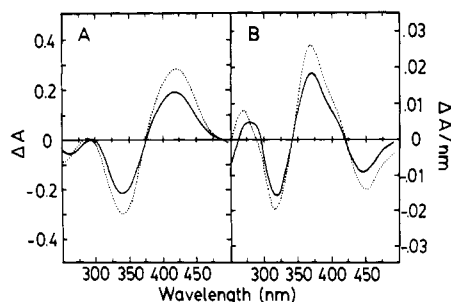


FIGURE 4: Difference spectra of the catalytic subunit and *N*-acetylcysteine modified with NBD-Cl. The reaction between NBD-Cl and 8  $\mu$ M C subunit (—) or 24  $\mu$ M *N*-acetylcysteine (---) was performed in split-cell cuvettes as described under Materials and Methods. (Panel A) Difference spectra; (panel B) first derivatives of the difference spectra from panel A.

centered at 420 nm indicates the formation of *S*-NBD-cysteine ( $\lambda_{\max} = 420$  nm; Birkett et al., 1970). The concurrent formation of a negative absorption band centered at 342 nm corresponds with the loss of free NBD-Cl ( $\lambda_{\max} = 343$  nm; Nitta et al., 1979). The extinction coefficient for *S*-NBD-cysteine at 420 nm of  $13\,000\text{ M}^{-1}\text{ cm}^{-1}$  (Birkett et al., 1970) was used to calculate a stoichiometry of  $1.9 \pm 0.1$  mol of NBD/mol of C subunit. This spectrum is very similar to that of *S*-NBD-*N*-acetylcysteine (Figure 4A), which also has a maximum at 420 nm and a minimum at 342 nm. Furthermore, the first derivatives of the difference spectra (Figure 4B) are identical. In contrast, the presence of *O*-NBD-*N*-acetyltyrosine ( $\lambda_{\max} = 385$  nm) shifts the maximum from 418 (at 10 *S*-NBD/*O*-NBD) to 390 nm (at 0.1 *S*-NBD/*O*-NBD). This difference spectral technique is sensitive enough to detect modification of 1 tyrosine per 12 cysteines ( $\lambda_{\max} = 418$  nm). Thus it is possible that NBD-Cl is modifying one tyrosine per six molecules of C subunit, or 0.17 mol of tyrosine/mol of C subunit. The presence of *N*-NBD is more easily detectable than the presence of *O*-NBD-tyrosine, since it has an absorption band centered at 475 nm ( $\epsilon = 26\,000\text{ M}^{-1}\text{ cm}^{-1}$ ; Aboderin et al., 1973) that stretches from 530 to 420 nm and produces a distinct shoulder on the difference spectrum (not shown). The limit of detection of *N*-NBD by our technique is approximately 0.07 mol of  $\text{NH}_2$ /mol of C subunit. No evidence was seen in these spectra for modification of tyrosine or amino groups. Although there may be modification of approximately 0.1 mol of tyrosine/mol of C subunit, which would escape detection by our technique, this amount of modification would not be expected to contribute significantly to the inactivation of the C subunit by NBD-Cl. To rule out the possibility that the peak at 420 nm might represent *O*-NBD-tyrosine or an *N*-NBD adduct with its  $\lambda_{\max}$  shifted due to its microenvironment, we denatured NBD-modified C with 5 M urea and recorded the difference spectrum. This spectrum (not shown) was the same as that in Figure 4, with a positive absorption band centered at 420 nm. Thus NBD-Cl inactivates the C subunit by modifying two cysteine residues.

**Stoichiometry of Inactivation.** To correlate the loss of phosphotransferase activity with degree of cysteine modification, we incubated C subunit with 0.24 mM [ $^{14}\text{C}$ ]NBD-Cl at pH 7.0. Portions were removed at specified times for determination of phosphotransferase activity and incorporation of radioactive NBD into protein. Complete inactivation is associated with modification of  $2.1 \pm 0.15$  mol of cysteine/mol of C subunit (Figure 5). This agrees well with the stoichiometry determined by difference spectroscopy. The linear correlation between [ $^{14}\text{C}$ ]NBD modification and loss of phosphotransferase activity suggests that both cysteines react

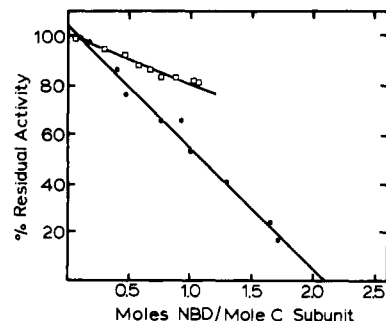


FIGURE 5: Inactivation of the catalytic subunit as a function of degree of modification by NBD-Cl. The catalytic subunit (0.5 mg/mL) was incubated with 0.24 mM [ $^{14}\text{C}$ ]NBD-Cl in 0.2 M Mops/10 mM  $\text{MgCl}_2$ /0.1 mM EDTA, pH 7.0, at 30 °C in the absence (●) or presence (□) of 2 mM ATP. Portions were removed at various times for assay of phosphotransferase activity and [ $^{14}\text{C}$ ]NBD incorporation into the catalytic subunit (methods A and B described under Materials and Methods). The aliquots removed for determination of phosphotransferase activity were diluted 1:25 with 0.2 M Mops (pH 7.0) immediately before assay.

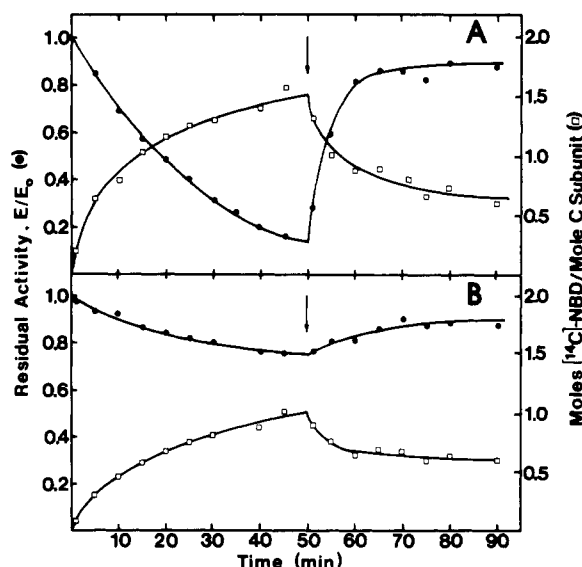


FIGURE 6: Time course of modification and inactivation of the catalytic subunit by [ $^{14}\text{C}$ ]NBD-Cl and reactivation by 2-mercaptoethanol. The catalytic subunit (0.5 mg/mL) was incubated with 0.24 mM [ $^{14}\text{C}$ ]NBD-Cl in the absence (panel A) or presence (panel B) of 2 mM ATP. Portions were analyzed for phosphotransferase activity (●) and [ $^{14}\text{C}$ ]NBD incorporation (□) as described in the legend to Figure 5. 2-Mercaptoethanol (100 mM final) was added at the times indicated by the arrows.

with NBD-Cl at similar rates. Inclusion of 2 mM ATP and 10 mM  $\text{MgCl}_2$  slows the rate of inactivation (Figure 6). One mole of cysteine per mole of C subunit, however, is modified with the loss of only 20–30% of phosphotransferase activity. Whether this inactivation is associated with modification of a unique cysteine or whether this represents modification of an average of 1 mol/mol of C requires additional experimentation.

**Effect of Chemical Modification of the C Subunit on Stoichiometry of [ $^{14}\text{C}$ ]NBD Reaction.** Additional evidence that NBD-Cl reacts only with thiol groups was provided by additional experiments with DTNB. Armstrong & Kaiser (1978) and Peters et al. (1977) reported that this reagent reacts with two or three thiol groups per C subunit, respectively, from the bovine heart type II enzyme. After the enzyme was treated with 0.2 mM DTNB, its catalytic activity was virtually abolished (Table III). NBD-Cl, moreover, failed to form a covalent adduct with this form of the enzyme. This is not due

Table III: Effects of Pretreatment of the Catalytic Subunit with Chemical Modification Reagents on Reaction with [ $^{14}$ C]NBD-Cl<sup>a</sup>

addition	enzyme activity [ $\mu$ mol/(min· mg)]	[ $^{14}$ C]NBD bound (mol/mol of enzyme)
none	19.7 $\pm$ 1.20	2.14 $\pm$ 0.17
DTNB, 0.2 mM	0.1 $\pm$ 0.01	0.05 $\pm$ 0.01
FSBA, 5.0 mM	1.2 $\pm$ 0.10	2.09 $\pm$ 0.16
acetonitrile, 10%	18.8 $\pm$ 1.20	2.04 $\pm$ 0.18

<sup>a</sup> The specified compounds were added to the catalytic subunit (0.37 mg/mL) in 0.2 M Mops (pH 7.0) and incubated at 30 °C for 30 min. Portions were removed for enzyme activity determinations as described under Materials and Methods with Ser-peptide as the substrate. Then [ $^{14}$ C]NBD-Cl was added to give a final concentration of 0.25 nM, and the incubation was continued for 30 min. Then 25- $\mu$ L portions were transferred to Whatman 3-MM filter paper strips, and incorporation of radioactivity was measured by method B as given under Materials and Methods. The means  $\pm$  SEM of three independent experiments are given. The FSBA was dissolved in acetonitrile to give a final concentration of this solvent of 10% during the incubation with the C subunit.

to consumption of NBD-Cl by 5-thio-2-nitrobenzoate formed during the reaction of DTNB with the C subunit because the amount generated during this reaction is only enough to consume about 7–8% of the added NBD-Cl (which is present in excess). This finding is consistent with the notion that NBD-Cl reacts solely with cysteine sulfhydryl groups in the C subunit. When the thiol groups are blocked, furthermore, other residues fail to form covalent adducts with NBD-Cl under the conditions of the experiment. This result confirms the identification of the modified residue by spectral analysis.

The possible effect of pretreatment with an active site directed reagent on the stoichiometry of the NBD-derivative formation was determined. FSBA forms a covalent adduct with a lysine residue in the porcine skeletal muscle type II subunit (Zoller & Taylor, 1979). This reagent and NBD-Cl exhibit hyperbolic concentration dependence of inactivation and MgATP protection against inhibition. We therefore presume that they are reacting with residues in the same site. Pretreatment of the enzyme with FSBA, however, fails to alter the stoichiometry or rate of the NBD modification of the C subunit. Furthermore, when FSBA (1–5 mM) is included during reaction of the C subunit with [ $^{14}$ C]NBD-Cl, it fails to alter the rate or extent of modification. This indicates that FSBA does not physically block the cysteines that are modified by NBD-Cl and suggests that the cysteines modified by NBD-Cl do not form part of the site on the C subunit occupied by FSBA.

**Reversal with 2-Mercaptoethanol.** Incubation of NBD-Cl-inactivated C subunit with excess 2-mercaptoethanol results in recovery of 85–90% of the original phosphotransferase activity. Under these conditions, the C subunit still retains 0.5 mol of [ $^{14}$ C]NBD/mol of enzyme (Figure 6A). ATP (2 mM), in the presence of 10 mM MgCl<sub>2</sub>, decreases the rates of C subunit inactivation and [ $^{14}$ C]NBD incorporation (Figure 6B). After 45 min, 1 mol of [ $^{14}$ C]NBD is bound per mol of C subunit, whereas the enzyme retains 75% of its original activity. After addition of 2-mercaptoethanol (100 mM final), phosphotransferase activity returns to about 90% of its original value. Nevertheless, as in the experiment in which ATP was omitted (Figure 6A), about 0.5 and 0.3 mol of [ $^{14}$ C]NBD remained covalently bound per mol of C subunit after incubation with 2-mercaptoethanol for 40 and 90 min, respectively. Similar results were obtained when dithiothreitol (10 mM) was used for reactivation. The incomplete removal of

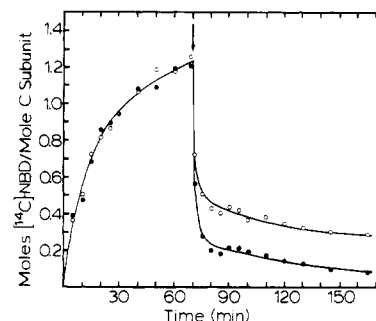


FIGURE 7: Effect of urea on removal of [ $^{14}$ C]NBD from the catalytic subunit by 2-mercaptoethanol. The catalytic subunit (41  $\mu$ g) was incubated in a final volume of 250  $\mu$ L with 0.1 mM [ $^{14}$ C]NBD-Cl at 22 °C in 50 mM Mops/0.1 M KCl, pH 7.0, and portions (10  $\mu$ L) were removed at the specified times and analyzed for [ $^{14}$ C]NBD incorporation by method B. At the time indicated by the arrow, portions (200  $\mu$ L) of 0.17 M 2-mercaptoethanol in buffer (O) or 200  $\mu$ L of 0.17 M 2-mercaptoethanol in 10 M urea (●) was added. Portions (25  $\mu$ L) were removed at the indicated times and analyzed by method B.

[ $^{14}$ C]NBD from the C subunit was not anticipated because rapid, complete thiolysis of the *S*-NBD adduct of (Na<sup>+</sup>-K<sup>+</sup>)ATPase (Cantley et al., 1978) and the *O*-NBD adduct of the mitochondrial ATPase (Ferguson et al., 1975a) have been reported. To examine the possibility that modification by NBD-Cl produces a conformational change that renders the *S*-NBD-cysteine less accessible to the solvent, we modified C subunit with [ $^{14}$ C]NBD-Cl and then reacted it with 100 mM 2-mercaptoethanol in the presence and absence of 6 M urea. NBD-modified C subunit contained 0.3 mol of bound [ $^{14}$ C]NBD after a 90-min incubation with 100 mM 2-mercaptoethanol (Figure 7). Inclusion of 6 M urea during incubation with 100 mM 2-mercaptoethanol resulted in faster and more complete removal of [ $^{14}$ C]NBD from the enzyme to a level of 0.08 mol/mol of C subunit after 90 min. These results suggest that the incomplete removal of NBD from modified C subunit is primarily due to steric hindrance of the approach of 2-mercaptoethanol, because denaturation of the enzyme with 6 M urea facilitates removal of NBD groups.

To determine whether thiolysis of NBD-inactivated C subunit by 2-mercaptoethanol results in reconstitution of native enzyme or whether the *S*-hydroxyethyl derivative is formed, we inactivated C subunit with NBD-Cl and reactivated it with 2-mercaptoethanol (as in Figure 6A). The reaction mixture was filtered on a Sephadex G-50 column to resolve the reactivated enzyme from the small molecules. Incubation of this enzyme with [ $^{14}$ C]NBD-Cl resulted in incorporation of 1.6 mol of [ $^{14}$ C]NBD/mol of C subunit with concomitant inactivation (results not shown). Thus thiolysis appears to proceed as follows: C-S-NBD + R-SH  $\rightleftharpoons$  C-SH + R-S-NBD, rather than the alternative C-S-NBD + R-SH  $\rightleftharpoons$  C-S-R + NBD-SH.

**Inactivation of Holoenzyme by NBD-Cl.** The effect of type II regulatory subunit from brain and type I and II regulatory subunits from bovine skeletal muscle on inactivation of C subunit by NBD-Cl was determined. Armstrong & Kaiser (1978) observed that in the absence of cAMP the rate of inactivation of the bovine heart type II holoenzyme by DTNB is much slower than the rate of inactivation of the free C subunit. In the presence of saturating concentrations of cAMP (0.5 mM), however, the rate of inactivation is greater. The time course of inactivation of free C subunit and brain and skeletal muscle holoenzymes (reconstituted from isolated subunits) by NBD-Cl in the absence and presence of cAMP is shown in Figure 8. In the absence of cAMP, R subunits

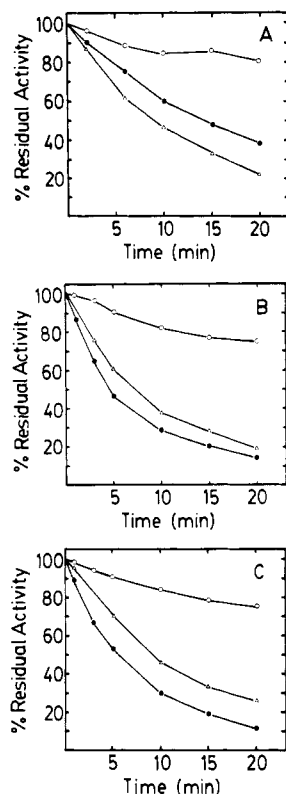


FIGURE 8: Inactivation of the catalytic subunit and holoenzyme by NBD-Cl. The catalytic subunit (20  $\mu\text{g/mL}$ ) was incubated with 50 mM Mops/0.1 M KCl, pH 7.0 ( $\Delta$ ), or regulatory subunit (30  $\mu\text{g/mL}$ ) in the presence ( $\bullet$ ) or absence ( $\circ$ ) of 0.6 mM cAMP for 20 min at 30  $^{\circ}\text{C}$ . NBD-Cl (30 mM in acetonitrile) was then added to give a final concentration of 0.3 mM. Portions were removed at the specified times and assayed as described under Materials and Methods. In the absence of NBD-Cl free C subunit retained 90% of the original activity, and holoenzyme retained greater than 95% of original activity. All values shown were corrected for this loss. (Panel A) Type II regulatory subunit from bovine brain; (panel B) type I regulatory subunit from bovine skeletal muscle; (panel C) type II regulatory subunit from bovine skeletal muscle.

(type II from brain and types I and II from skeletal muscle) slow the rate of inactivation of the C subunit by NBD-Cl. Under conditions in which free C subunit is inhibited by 75%, the holoenzymes from skeletal muscle or brain are inhibited only 20%. When 0.6 mM cAMP is present during incubation with NBD-Cl, however, the brain and skeletal muscle holoenzymes behave quite differently. Both type I and type II R subunits from skeletal muscle accelerate the rate of inactivation of the C subunit by NBD-Cl. This result is similar to that reported by Armstrong & Kaiser (1978) for the bovine cardiac muscle enzyme. The type II R subunit from brain, however, slows the rate of inactivation of the C subunit by NBD-Cl. This result was obtained on three independent R and C preparations. Thus the type II R subunit from bovine brain, unlike the type I and type II R subunits from skeletal muscle, decreases the reactivity of sulfhydryl groups associated with inactivation even in the presence of high concentrations of cAMP. Erlichman et al. (1974) reported that autophosphorylation of type II holoenzyme facilitates dissociation. Using gel filtration, Armstrong & Kaiser (1978) determined that 0.5 mM cAMP completely (>99.5%) dissociates bovine heart type II dephosphoholoenzyme. However, Erlichman et al. (1980) reported that type II R subunit from bovine brain has a lower affinity for cAMP than does skeletal muscle type II R subunit. In experiments under the conditions described in Figure 8, we determined that skeletal muscle type II holoenzyme activity is maximally stimulated at approximately

1  $\mu\text{M}$  cAMP, while brain holoenzyme activity is maximally stimulated at approximately 10  $\mu\text{M}$  cAMP; raising the cAMP concentrations above these levels fails to further stimulate activity. Thus, the partial protection against NBD-Cl inactivation afforded by bovine brain R (which is probably a mixture of phospho and dephospho forms) in the presence of 0.6 mM cAMP is not due to incomplete dissociation of the holoenzyme. This fact suggests that R and C subunits communicate in solution even in the presence of high levels of cAMP as proposed by Armstrong & Kaiser (1978).

## Discussion

NBD-Cl inactivates the isolated catalytic subunit of bovine brain cAMP-dependent protein kinase by covalent modification. This process appears to occur at or near the active site as suggested by the following lines of evidence. (1) The reaction between NBD-Cl and the C subunit follows pseudo-first-order kinetics, and the rate of inactivation displays a hyperbolic dependence on NBD-Cl concentration (Figure 2), indicating a saturable site of action of NBD-Cl. A double-reciprocal plot ( $1/k_{\text{app}}$  vs.  $1/[\text{NBD-Cl}]$ ; Figure 2) is linear with a nonzero ordinate intercept, suggesting the formation of a noncovalent complex before covalent modification, as depicted in eq 1. The nature of such an enzyme-NBD-Cl complex, however, is unknown. (2) Substrates and competitive inhibitors that bind at the active site of the C subunit protect against inactivation (Table II). Surprisingly, adenosine protects against inactivation more effectively than 5'-AMP (when 1 mM concentrations are used). Using steady-state inhibition of phosphotransferase activity, we determined the  $K_i$  for 5'-AMP to be  $745 \pm 10 \mu\text{M}$ , which is much higher than the  $K_i$  values for adenosine ( $42 \pm 3 \mu\text{M}$ ) and ADP ( $13 \pm 3 \mu\text{M}$ ). Thus, the ability of a nucleotide or nucleoside to protect against inactivation by NBD-Cl is directly related to its affinity for the nucleotide binding site on the C subunit. Adenosine competitively inhibits the rate of reaction of NBD-Cl with the C subunit (Figure 3). The  $K_A$  for adenosine of 44  $\mu\text{M}$  determined by plotting the data in Figure 3 according to eq 4 agrees closely with the value of 42  $\mu\text{M}$  determined by steady-state inhibition of phosphotransferase activity and suggests that NBD-Cl and adenosine both act at the same site on the C subunit, namely, the nucleotide binding domain as described by eq 1 and 3. The results outlined above are consistent with criteria set forth for processes occurring at enzyme active sites (Wold, 1977; Meloche, 1967).

Protein substrates and competitive inhibitors with respect to protein substrates fail to protect against inactivation by NBD-Cl. Histone IIa (2 mg/mL) actually accelerates the rate of inactivation. This phenomenon was recently observed by Hixson & Krebs (1979) for FSBA, a reagent specific for adenine nucleotide binding sites (Pal et al., 1975; Wyatt & Colman, 1977), which modifies an active site lysine in porcine skeletal muscle C subunit (Zoller & Taylor, 1979). Hixson and Krebs attributed the accelerated rate of inactivation to the large net positive charge of the histones; however, it may also be due to a conformational effect resulting from histone binding.

The difference spectrum of C subunit modified by NBD-Cl has an absorption band characteristic of S-NBD-cysteine centered at 420 nm (Birkett et al., 1970). The magnitude of the peak corresponds to modification of 1.9 mol of cysteine/mol of C subunit. This stoichiometry is confirmed by experiments with [ $^{14}\text{C}$ ]NBD-Cl (Figure 5) that show a parallel relationship between the degree of modification by NBD-Cl and the extent of inactivation; complete inactivation results from modification of  $2.1 \pm 0.15$  mol of cysteine/mol of C



subunit. Kupfer et al. (1979) reported that  $2.3 \pm 0.1$  mol of DTNB react with 1 mol of C subunit from rabbit skeletal muscle in three kinetically distinct phases, with only one of the cysteines being important for activity. They suggested that the C subunit contains three sulfhydryls, one of which is at or near the active site. The presence of 3 mol of cysteine/mol of C subunit has been reported for type I C subunit from rabbit skeletal muscle (Bechtel et al., 1977) and type II C subunit from bovine heart (Peters et al., 1977). Peters et al. (1977) determined this value for the bovine heart enzyme by amino acid analysis and by reaction of enzymic thiols with DTNB. They found that complete inactivation was associated with the reaction of 3.0 mol of DTNB/mol of C subunit. In contrast to this, Armstrong & Kaiser (1978) reported that complete inactivation of bovine heart C subunit is associated with the modification of 2 mol of cysteine/mol of C subunit, and the maximum reaction they observed was  $2.1 \pm 0.1$  mol of DTNB/mol of C subunit. The stoichiometry of  $2.1 \pm 0.15$  mol of NBD-Cl/mol of C subunit that we observed agrees well with the value of Armstrong & Kaiser (1978). These investigators suggested that their observed stoichiometry might be due to oxidation of a cysteine during purification of the C subunit. Shoji et al. (1981), however, presented the complete amino acid sequence of type II C subunit from bovine heart. This enzyme, which was purified by the method of Peters et al. (1977), contains 2 mol of cysteine/mol of C subunit as determined by sequence analysis.

Of the two enzymic cysteines that react with NBD-Cl, one appears to be more important for activity than the other. That finding is documented by experiments in which MgATP is included during reaction of C subunit with NBD-Cl (Figures 5 and 6) and when inhibited enzyme is reactivated with 2-mercaptoethanol (Figure 6). When MgATP is present during reaction with NBD-Cl, 1 mol of cysteine on the average is modified per mol of C subunit with the loss of only 20–25% of phosphotransferase activity and 2-mercaptoethanol-reactivated enzyme, which contains 0.4–0.5 mol of NBD/mol, is 90% active. MgATP does not afford complete protection from inactivation by NBD-Cl; inactivation of the C subunit occurs slowly in the presence of millimolar concentrations of ATP. Also, the ATP analogue 5'-[p-(fluorosulfonyl)benzoyl]-adenosine (FSBA) does not alter the rate or extent of modification of cysteines by NBD-Cl. Two main possibilities exist for the mechanism of protection by MgATP. The first involves direct steric hindrance to the approach of NBD-Cl. If this is the mechanism, then the failure of FSBA to protect against inactivation suggests that FSBA binds to the C subunit such that it does not block the cysteines as MgATP, MgADP, or adenosine might. The second mechanism for protection by MgATP involves a conformational change of the C subunit subsequent to binding of ligand, which buries or otherwise renders at least one of the cysteines less reactive toward NBD-Cl. On the basis of circular dichroism studies, Sugden et al. (1976) reported that MgATP induces a conformational change in the C subunit. If this were the mechanism by which MgATP protects against NBD modification, then the lack of protection by FSBA suggests that this analogue fails to elicit the conformational change necessary for protection against NBD modification.

In the absence of cAMP, the rates of inactivation of type I and II holoenzymes from bovine skeletal muscle and type II holoenzyme from bovine brain are much slower than the inactivation of the free C subunit. The R subunits thus render at least one of the two C subunit cysteines less reactive toward NBD-Cl. When the bovine brain holoenzyme is reacted with

NBD-Cl in the presence of 0.6 mM cAMP, the rate of inactivation is slightly slower than the rate of inactivation of the free C subunit (Figure 8). This slowing is not due simply to consumption of NBD-Cl by the R subunit. This is supported by two lines of evidence: (1) NBD-Cl is present in a large excess, and the amount of R subunit added is enough to react with only about 1% of the NBD-Cl present; (2) skeletal muscle R subunits (both types I and II), which also react with NBD-Cl, accelerate the rate of inactivation of the C subunit under identical conditions (Figure 8). Because 0.5 mM cAMP completely (>99.5%) dissociates both phospho- and dephosphoholoenzymes from bovine heart (Armstrong & Kaiser, 1978) and cAMP concentrations above 10  $\mu$ M maximally stimulate activity of both skeletal muscle and brain holoenzymes (unpublished observations), this result is not due to the presence of undissociated holoenzyme; rather it suggests that the R and C subunits are interacting despite the high concentration of cAMP in the solution. Armstrong & Kaiser (1978) reported that in the presence of 0.5 mM cAMP the rates of inactivation by DTNB are as follows: dephosphoholoenzyme > phosphoholoenzyme > free C subunit. They interpreted these results in terms of ternary complex in which one or both of the thiol groups is activated toward reaction with DTNB. The different effects of  $R_2(cAMP)_4$  on inactivation we observed could be due to one or both of the following possibilities [expressed in terms of  $R_2(cAMP)_4$  complexes]. First, the postulated  $R_2(cAMP)_4C_2$  complexes from cardiac or skeletal muscle and brain may be identical, but the sulfhydryls could be activated toward disulfide exchange with DTNB but exhibit reduced activity toward nucleophilic reaction with NBD-Cl. Second, the  $R_2(cAMP)_4C_2$  complexes from cardiac or skeletal muscle and brain may differ in their interaction with the C subunit. Fleischer et al. (1976) and Ehrlichman et al. (1980), for example, reported that bovine brain R subunit differs immunochemically from the bovine heart R subunit. Furthermore, Ehrlichman et al. (1980) suggested that type II R subunits are divided into two distinct subclasses, neural-specific and nonneural protein kinases, which differ in their antigenic determinants. The observation that the brain and skeletal muscle complexes behave differently toward the same reagent (NBD-Cl) provides a second, independent confirmation of differences between the neural and nonneural type II protein kinases.

## References

- Aboderin, A. A., Boedefeld, E., & Luisi, P. L. (1973) *Biochim. Biophys. Acta* 328, 20.
- Armstrong, R. N., & Kaiser, E. T. (1978) *Biochemistry* 17, 2840.
- Beavo, J. A., Bechtel, P. J., & Krebs, E. G. (1975) *Adv. Cyclic Nucleotide Res.* 5, 241.
- Bechtel, P. J., Beavo, J. A., & Krebs, E. G. (1977) *J. Biol. Chem.* 252, 2691.
- Birkett, D. J., Price, N. C., Radda, G. K., & Salmon, A. G. (1970) *FEBS Lett.* 6, 346.
- Cantley, L. C., Gelles, J., & Josephson, L. (1978) *Biochemistry* 17, 418.
- Corbin, J. D., Sugden, P. H., West, L., Flockhart, D. A., Lincoln, T. M., & McCarthy, D. (1978) *J. Biol. Chem.* 253, 3997.
- Ehrlichman, J., Rosenfeld, R., & Rosen, O. M. (1974) *J. Biol. Chem.* 249, 5000.
- Ehrlichman, J., Sarkar, D., Fleischer, N., & Rubin, C. S. (1980) *J. Biol. Chem.* 255, 8179–8184.
- Ferguson, S. J., Lloyd, W. J., Lyons, J. H., & Radda, G. K. (1975a) *Eur. J. Biochem.* 54, 117.



- Ferguson, S. J., Lloyd, W. J., & Radda, G. K. (1975b) *Eur. J. Biochem.* 54, 127.
- Fleischer, N., Rosen, O. M., & Reichlin, M. (1976) *Proc. Natl. Acad. Sci. U.S.A.* 73, 54.
- Ghosh, P. B., & Whitehouse, M. W. (1968) *Biochem. J.* 108, 155.
- Gill, G. N., & Garren, L. D. (1970) *Biochem. Biophys. Res. Commun.* 39, 335.
- Glass, D. B., Masaracchia, R. A., Feramisco, J. R., & Kemp, B. E. (1978) *Anal. Biochem.* 87, 566.
- Hartl, F. T. (1980) *Fed. Proc., Fed. Am. Soc. Exp. Biol.* 39, 2094.
- Hartl, F. T., & Roskoski, R., Jr. (1979) *Int. Cong. Biochem., XIth, Abstr.*, 259.
- Hixson, C. S., & Krebs, E. G. (1979) *J. Biol. Chem.* 254, 7509.
- Kitz, R., & Wilson, I. B. (1962) *J. Biol. Chem.* 237, 3245.
- Kupfer, A., Gani, V., Jimenez, J. S., & Shaltiel, S. (1979) *Proc. Natl. Acad. Sci. U.S.A.* 76, 3073.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem.* 193, 265.
- Meloche, H. P. (1967) *Biochemistry* 6, 2273.
- Muneyama, L., Bauer, J. J., Shuman, D. A., Robins, R. K., & Simon, L. N. (1971) *Biochemistry* 10, 2390.
- Nitta, K., Brachter, S. C., & Dronman, J. J. (1979) *Biochem. J.* 117, 385.
- Pal, P. K., Wechter, W. J., & Colman, R. F. (1975) *J. Biol. Chem.* 250, 8140.
- Peters, K. A., Demaille, J. G., & Fischer, E. H. (1977) *Biochemistry* 16, 5691.
- Rosen, O. M., & Erlichman, J. (1975) *J. Biol. Chem.* 250, 7788.
- Shoji, S., Parmelee, D. C., Wade, R. D., Kumar, S., Ericsson, L. H., Walsh, K. A., Neurath, H., Long, G. L., Demaille, J. G., Fischer, E. H., & Titani, K. (1981) *Proc. Natl. Acad. Sci. U.S.A.* 78, 848.
- Sugden, P. H., Holladay, L. A., Reimann, E. M., & Corbin, J. D. (1976) *Biochem. J.* 159, 409.
- Tao, M., Salas, M. L., & Lipmann, F. (1970) *Proc. Natl. Acad. Sci. U.S.A.* 67, 408.
- Weber, W., Vogel, C., & Hiltz, H. (1979) *FEBS Lett.* 99, 62.
- Witt, J. J., & Roskoski, R., Jr. (1975a) *Biochemistry* 14, 4503.
- Witt, J. J., & Roskoski, R., Jr. (1975b) *Anal. Biochem.* 66, 253.
- Wold, F. (1977) *Methods Enzymol.* 46, 3.
- Wyatt, J. L., & Colman, R. F. (1977) *Biochemistry* 16, 1333.
- Zoller, M. J., & Taylor, S. S. (1979) *J. Biol. Chem.* 254, 8363.

## Substrate Specificity of the Collagenolytic Serine Protease from *Uca pugilator*: Studies with Collagenous Substrates<sup>†</sup>

Howard G. Welgus, Gregory A. Grant,\* John J. Jeffrey, and Arthur Z. Eisen

**ABSTRACT:** The collagenolytic protease from *Uca pugilator* was studied with respect to its catalytic properties on collagen types I-V. The crab protease degraded all five collagen types, producing multiple cleavages in the triple helix of each native collagen at 25 °C. The major early cleavage in the  $\alpha 1$  polypeptide chain of collagen types I-III occurred at a  $3/4:1/4$  locus, resulting in fragments electrophoretically similar to the TC<sup>A</sup> and TC<sup>B</sup> products of mammalian collagenase action. Interestingly, a propensity toward this same cleavage was observed even following thermal denaturation of the substrates. The ability of the crab protease to degrade all native collagen types and to catalyze cleavages at multiple loci in the triple

helix distinguishes its action from that of mammalian collagenases. The collagenolytic activity of the crab protease was also examined on fibrillar collagen and compared to that of human skin fibroblast collagenase. Enzyme concentrations of fibroblast collagenase which resulted in the saturation of available substrate sites failed to show such an effect in the case of the crab protease. Binding studies of the crab protease to fibrillar collagen likewise indicated substantially reduced levels of enzyme binding in comparison to fibroblast collagenase. These data suggest that the affinity of the crab protease for native collagen is considerably less than the affinity of mammalian collagenase for this substrate.

The fiddler crab, *Uca pugilator*, is a predacious scavenger that feeds on animal tissues frequently containing collagen as a major constituent protein. A collagenolytic protease isolated from the hepatopancreas of this crustacean has been purified to homogeneity (Eisen et al., 1973) and shown by the determination of the complete covalent structure to be a serine protease (Grant et al., 1980) homologous to the pancreatic serine proteases of vertebrates. This crab protease is a good

general protease, displaying a broader peptide bond specificity than either trypsin or chymotrypsin on noncollagenous substrates (Grant & Eisen, 1980). In addition, the enzyme has been shown to cleave the native triple helix of type I collagen in the area of the TC<sup>A</sup><sub>75</sub> locus (Eisen & Jeffrey, 1969; Eisen et al., 1973). Not surprisingly, this protease also effects a rapid conversion of intramolecular cross-linked  $\beta$  dimers of collagen to monomeric  $\alpha$  chains, indicating an additional site of cleavage at the nonhelical ends of the collagen molecule (Eisen & Jeffrey, 1969). While the crab protease was the first example of a serine protease with significant collagenolytic activity, similar enzymes have now been reported from the dog pancreas (Takahashi & Seifter, 1974), the fungus *Entomophthora coronata* (Hurion et al., 1979), and the insect *Hypoderma*

<sup>†</sup> From the Division of Dermatology, Department of Medicine and Department of Biological Chemistry, Washington University School of Medicine, St. Louis, Missouri 63110. Received April 12, 1982. This work was supported by U.S. Public Health Service Grants AM 31078, GM 27784, AM 12129, and TO-AM 07284.