Chambers, R. W., & Khorana, H. G. (1958) *J. Am. Chem. Soc. 80*, 3749.
Chavane, V. (1949) *Ann. Chim. (Paris) 4 (12)*, 383.
Cottam, G. L., Kupiecki, F. P., & Coon, M. J. (1968) *J. Biol. Chem. 243*, 1630.
Dougherty, T. M. (1982) Ph.D. Dissertation, University of Wisconsin.
Gibbons, B. H., & Edsall, J. T. (1963) *J. Biol. Chem. 238*, 3502.
Jencks, W. P., & Gilchrist, M. (1965) *J. Am. Chem. Soc. 87*, 3199.
Kupiecki, F. P., & Coon, M. J. (1960) *J. Biol. Chem. 235*, 1944.
Meyerhof, O., & Lohmann, K. (1928) *Biochem. Z. 196*, 49.

Adenosine Cyclic 3′,5′-Monophosphate Dependent Protein Kinase: Nucleotide Binding to the Chemically Modified Catalytic Subunit†

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ABSTRACT: 5′-[*p*-(Fluorosulfonyl)benzoyl]adenosine (FSBA) inactivates the catalytic subunit of the adenosine cyclic 3′,5′-monophosphate dependent protein kinase isolated from bovine cardiac muscle by covalent modification of lysine-71, whereas 7-chloro-4-nitro-2,1,3-benzoxadiazole (NBD-Cl) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) react with cysteines-199 and -343 to inactivate the enzyme. All three of these reagents have been postulated to modify residues at or near the active site of the catalytic subunit. ATP (2 mM) in the presence of excess Mg^{2+} (10 mM) protects the enzyme against inactivation by these reagents. AMP did not afford any protection, but adenosine slightly decreased the rate of inactivation. The specific effects of covalent modification of lysine-71 and cysteines-199 and -343 on nucleotide binding were characterized by fluorescence-polarization titrations with *lin*-benzoadenine nucleotides as fluorescent ligands. *lin*-Benzoadenosine is a competitive inhibitor of the catalytic subunit with respect to ATP with a K_i (38 μM) similar to the K_i for adenosine (35 μM). This value agrees well with the K_d (32 μM) for adenosine determined by fluorescence-polarization titrations. *lin*-Benzoadenosine 5′-diphosphate (*lin*-benzo-ADP) has been shown to be a competitive inhibitor with respect to ATP [Hartl, F. T., Roskoski, R., Jr., Rosendahl, M. S., & Leonard, N. J. (1983) *Biochemistry 22*, 2347], and *lin*-benzoadenosine 5′-triphosphate (*lin*-benzo-ATP) is a substrate for the phosphotransferase activity of the protein kinase. Chemical modification by FSBA, NBD-Cl, or DTNB resulted in greater than 85% inhibition of phosphotransferase activity as well as complete inhibition of *lin*-benzo-ADP and *lin*-benzo-ATP binding in the presence of 10 mM Mg^{2+}. *lin*-Benzoadenosine, on the other hand, bound to the enzyme with the same K_d and stoichiometry (1 mol/mol) as it did to the unmodified enzyme (K_d, 26–35 μM). While all effectively displaced *lin*-benzoadenosine bound to the unmodified catalytic subunit, AMP—but not MgATP or MgADP—displaced the fluorescent probe from enzyme modified with NBD-Cl, DTNB, or FSBA. The K_d for AMP (804–856 μM), however, was 25% greater for the modified enzyme. These reagents, which are thought to modify residues that are at or near the active site of the catalytic subunit, inactivate the enzyme by inhibiting nucleotide binding. This effect involves the region on the C subunit complementary to the β- and γ-phosphates of the ATP molecule as compared to the region complementary to the α-phosphate of the nucleotide binding portion of the C subunit.

Adenosine cyclic 3′,5′-monophosphate (cAMP)[1] dependent protein kinases (ATP:protein phosphotransferase, EC 2.7.1.37) catalyze the phosphorylation of polypeptidic serine and threonine residues. The enzyme is composed of dissimilar regulatory and catalytic subunits. The complete amino acid sequences of the bovine cardiac muscle type II regulatory subunit (Takio et al., 1982) and catalytic subunit (Shoji et al., 1981, 1983) are known. The enzyme has several distinct functional sites that contribute to overall enzyme activity. These sites include an ATP binding site, a protein substrate binding site, and a site(s) for divalent cations. Each has been studied by a variety of approaches. Armstrong et al. (1979a), Granot et al. (1979, 1980), and Rosevear et al. (1983) have utilized nuclear magnetic resonance and electron spin resonance to study the interaction of nucleotides, synthetic peptides, and divalent cations with the catalytic subunit and to characterize the conformation of the bound nucleotide. The binding constants for a series of nucleotide analogues have been determined in an effort to characterize the ATP binding site of the catalytic subunit (Hoppe et al., 1977, 1978; Bhatnagar et al., 1983).

Possible amino acid residues important to enzyme function have been assessed by chemical modification studies. A variety of sulfhydryl-directed reagents (NBD-Cl, DTNB, and iodoacetic acid) inhibit the ATP:protein phosphotransferase activity of the catalytic subunit, suggesting that a cysteine residue is situated at or near the active site (Sugden et al., 1976; Bechtel

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[1] Abbreviations: cAMP, adenosine cyclic 3′,5′-monophosphate; R, regulatory subunit of cAMP-dependent protein kinase; C, catalytic subunit of cAMP-dependent protein kinase; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); FSBA, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine; Ser-peptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly; MOPS, 3-(*N*-morpholino)propanesulfonic acid; SDS, sodium dodecyl sulfate; AMP-PNP, adenosine 5′-(β,γ-imidotriphosphate).

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et al., 1977; Peters et al., 1977; Armstrong & Kaiser, 1978; Nelson & Taylor, 1981, 1983; Hartl & Roskoski, 1982). The affinity label FSBA was shown to inactivate the C subunit (Hixson & Krebs, 1979) by modifying a single lysine residue (Zoller & Taylor, 1979; Zoller et al., 1981). Witt & Roskoski (1975) demonstrated inhibition of the catalytic subunit with ethoxyformic anhydride and suggested the involvement of a tyrosine residue.

In order to identify amino acid residues that may contribute to nucleotide binding, we have used a fluorescence-polarization titration technique for characterizing the ATP binding site of the catalytic subunit of the bovine cardiac muscle type II cAMP-dependent protein kinase. We have used fluorescent *lin*-benzoadenine nucleotides, "stretched-out" analogues of ATP (Leonard et al., 1978), for the determination of nucleotide binding to the chemically modified as well as unmodified C subunit.

Materials and Methods

The synthetic heptapeptide (Ser-peptide) used as substrate (Leu-Arg-Arg-Ala-Ser-Leu-Gly) was purchased from Boehringer Mannheim Biochemicals. Carrier-free [γ-^{32}P]ATP was purchased from ICN. The salts of divalent metal ions were purchased from Aldrich Chemical Co. All other chemicals were purchased from Sigma Chemical Co. The concentrations of *lin*-benzoadenine nucleoside and nucleotides were measured by absorbance at 331 nm using an extinction coefficient of 9750 M^{-1} cm^{-1} (Leonard et al., 1976).

Protein Kinase Preparation and Activity Measurements. The type II catalytic subunit from bovine cardiac muscle was purified by the procedure described by Hartl & Roskoski (1982). Enzyme activity was measured radioisotopically as described by Roskoski (1983) in triplicate at 23 °C. The Ser-peptide (K_m, 10 μM; Cook et al., 1982) and ATP (K_m, 10 μM; Cook et al., 1982) concentrations were 100 and 200 μM, respectively, in the assay mixtures. The C subunit concentration was 7–10 nM. Protein concentrations were determined by the procedure of Lowry et al. (1951) or Bradford (1976) using bovine serum albumin or ovalbumin as the standard, respectively.

Chemical Modification of the Catalytic Subunit. The bovine cardiac muscle C subunit was chemically modified with either NBD-Cl, DTNB, or FSBA as follows.

(*a*) *NBD-Cl.* The catalytic subunit (0.01–1 mg/mL) was incubated with 0.5 mM NBD-Cl in 0.2 M MOPS (pH 7.0) at 30 °C for 30 min. NBD-Cl was added as a freshly prepared stock solution in acetonitrile. Because NBD-Cl is light sensitive, stock solutions and reaction mixtures were protected from light. The final concentration of acetonitrile in the reaction mixtures was 1–5%; these concentrations failed to alter C subunit activity significantly. Portions were removed throughout the course of the reaction and assayed for C subunit activity as previously described. [^{14}C]NBD incorporation in the catalytic subunit following incubation with [^{14}C]NBD-Cl was determined as described by Hartl & Roskoski (1982).

(*b*) *DTNB.* The catalytic subunit (0.01–1 mg/mL) was incubated with 0.25 mM DTNB in 0.2 M MOPS (pH 7.0) at 24 °C for 30 min. Portions were then removed at various times for phosphotransferase activity determinations.

(*c*) *FSBA.* Inhibition of the catalytic subunit was carried out at 37 °C in 0.2 M MOPS (pH 7.0). The reaction was initiated by the addition of FSBA to a final concentration of 1 mM. FSBA was prepared in dimethyl sulfoxide and the final concentration in the reaction mixture was 1–5%. These concentrations did not affect C subunit activity. Aliquots (5–10 μL) were removed at regular intervals and assayed for ATP:protein phosphotransferase activity as described above.

To separate modified enzyme from excess unreacted reagent, reaction mixtures were cooled to 4 °C and chromatographed on a 1 × 14 cm column of Sephadex G-50 (fine). Fractions (10 drops, approximately 0.5 mL) were collected. Radioactivity was measured in samples (5–10 μL) from each fraction when [^{14}C]NBD-Cl was used, and the absorbance at 280 nm was measured when nonradioactive reagents were used. The covalently modified C subunit could be separated from excess inhibitor in less than 30 min by this procedure.

Fluorescence-Polarization Titrations. The fluorescence measurements were performed with an SLM 4800 spectrofluorometer interfaced with a Hewlett-Packard HP 9825A calculator. Fluorescence polarization was calculated according to a program supplied by SLM Instruments, Inc. Polarization (P) and anisotropy (A) are defined as follows:

$$P = \frac{I_\parallel - I_\perp}{I_\parallel + I_\perp} \qquad A = \frac{I_\parallel - I_\perp}{I_\parallel + 2I_\perp}$$

where $I_\parallel$ and $I_\perp$ are the intensities observed parallel and perpendicular to the polarization of the exciting light, respectively. Polarization and anisotropy values of the *lin*-benzoadenine nucleotides were determined by using calcite polarizers. Excitation was at 334 nm, with 4-nm resolution, and emitted light was isolated with a Schott KV 389 filter.

Three types of polarization titrations were performed in order to determine the binding constants of various nucleotides for the catalytic subunit. The titrations were performed in 50 mM MOPS (pH 7.0) and 100 mM NaCl.

(*a*) *Dilution Titration.* Polarization as a function of varying C subunit concentration (at constant *lin*-benzoadenine nucleotide) was performed as described in our earlier reports (Hartl et al., 1983; Bhatnagar et al., 1983) to determine P_b. P_b is the polarization value when all *lin*-benzoadenine nucleotide is bound to the catalytic subunit (at infinite C subunit concentration). The theoretical polarization of *lin*-benzoadenosine rigidly bound to protein kinase was calculated from Perrin's equation (Perrin, 1926) as described earlier for *lin*-benzo-ADP (Hartl et al., 1983). *lin*-Benzoadenosine has a fluorescence lifetime of 3.7 ns (Scopes et al., 1977). The value of P_0, the intrinsic polarization of the fluorophore, for *lin*-benzoadenosine was determined by measuring fluorescence polarization in solutions of varying viscosities (η) made with sucrose or glycerol; $1/P$ was plotted vs. T/η and $1/P_0$ was obtained from the ordinate intercept where $\eta \rightarrow \infty$. We have previously used the same method (Hartl et al., 1983) to obtain the value of P_0 for *lin*-benzo-ADP at 334 nm.

(*b*) *Addition Titration.* Polarization (P_{obsd}) was recorded as described previously (Hartl et al., 1983; Bhatnagar et al., 1983) at each nucleotide concentration after addition of successive increments of *lin*-benzoadenine nucleotide with a constant C subunit concentration. The dissociation constant (K_d) of *lin*-benzoadenine nucleotide for the C subunit was then determined by Scatchard analysis (Scatchard, 1949). The average angle of rotation (ω) of *lin*-benzoadenosine rigidly bound to the catalytic subunit was calculated from the equation of Perrin (1929) as determined previously for *lin*-benzo-ADP by Hartl et al. (1983).

(*c*) *Displacement Titration.* The dissociation constant (K_d) of various nucleotide analogues for the C subunit was determined by displacing the fluorescent *lin*-benzoadenine nucleotide bound to the C subunit with other nucleotides. A description for the determination of such a K_d has been presented earlier (Bhatnagar et al., 1983). A typical dis-

placement titration with *lin*-benzoadenosine as the fluorescent probe was performed as follows. A low concentration (1–2 μM) of *lin*-benzoadenosine (to give a polarization, P_{max}, of about 0.10–0.11) was added to the 4–6 μM covalently modified or unmodified (control) catalytic subunit in a total volume of 150 μL (in a 3 × 3 × 24 mm cuvette). The fluorescence polarization was recorded (P_{max}). $MgSO_4$ (10 mM final concentration) was then added in a volume of 2 μL to measure any additional enhancement in polarization. No significant change in polarization was observed on addition of the divalent metal ion, indicating that *lin*-benzoadenosine bound to the enzyme in the absence of the metal ion. *lin*-Benzo-ADP binding, on the other hand, was dependent on divalent metal ion (Bhatnagar et al., 1983). Then, increasing concentrations of the competing ligand were added (1 μL at a time), which displaced some of the *lin*-benzoadenosine bound to the enzyme. After equilibration, the polarization following each addition was then recorded (P_{obsd}). The minimum polarization (P_f) corresponding to that of free or unbound *lin*-benzoadenosine (when no ligand is bound to the C subunit) was determined by completely displacing *lin*-benzoadenosine bound to the unmodified C subunit with ATP. This value was in good agreement with the P_f observed in an addition titration with *lin*-benzoadenosine. There was a maximum of 2% difference between P_f values recorded on different days. Bound *lin*-benzoadenosine and free *lin*-benzoadenosine were calculated from P_{max}, P_f, and P_{obsd} at each concentration of the displacing ligand as described in Bhatnagar et al. (1983).

K_d values were also calculated on the basis of anisotropy. The maximum difference between these values obtained from polarization and anisotropy was within experimental error (less than 2%). This indicates that the polarization is additive. Our data are based on polarization values as a matter of convenience.

Results

Interaction of lin-Benzoadenosine with the Catalytic Subunit. *lin*-Benzoadenosine is an inhibitor of catalytic subunit activity (Schmidt et al., 1978). If *lin*-benzoadenosine interacts with the nucleotide portion of the active site, then it would be expected to be a competitive inhibitor with respect to MgATP. The catalytic subunit was therefore incubated with varying ATP concentrations at a fixed, saturating Ser-peptide concentration (150 μM; Cook et al., 1982) in the presence of various fixed concentrations of *lin*-benzoadenosine. As shown in Figure 1, *lin*-benzoadenosine is a competitive inhibitor with respect to MgATP. A slope vs. *lin*-benzoadenosine replot (Figure 1, inset) is linear and yields a K_i for *lin*-benzoadenosine of 37.4 ± 4.1 μM. This corresponds well with our previously determined K_i for adenosine of 35 μM (Cook et al., 1982). Thus *lin*-benzoadenosine, which is 2.4 Å wider in the adenine moiety, binds to the active site of the catalytic subunit as well as does adenosine.

Fluorometric titrations were performed for measurement of the interaction of *lin*-benzoadenosine with the catalytic subunit. No difference was observed between the fluorescence yields of the emission spectra of *lin*-benzoadenosine in the presence and absence of the catalytic subunit (not shown). We previously made a similar observation for *lin*-benzo-ADP (Hartl et al., 1983). Binding to the enzyme, however, is accompanied by an increase in polarization. The binding of *lin*-benzoadenosine to the bovine heart free catalytic subunit was measured by fluorescence-polarization dilution and addition titrations as described under Materials and Methods.

The polarization (P_b) of *lin*-benzoadenosine bound to the catalytic subunit was determined by dilution titration. Extrapolation of a plot of (polarization)$^{-1}$ vs. [protein kinase]$^{-1}$ to zero resulted in a value of P_b of 0.291 ± 0.011. This corresponds well with a P_b value of 0.306 calculated by the Perrin equation (Perrin, 1926) for rigidly bound *lin*-benzoadenosine. Furthermore, the calculation of the average angle of rotation (ω) of *lin*-benzoadenosine bound to the catalytic subunit during the lifetime of its excited state (3.7 ns; Scopes et al., 1977) yielded a value of 23.2°. This value is very close to the theoretical value of 24° calculated from the Perrin equation (Perrin, 1929) for tightly bound fluorescent ligand. P_0 (the intrinsic polarization of the fluorophore) and P_b (the polarization of the fluorophore in the bound state) are similar within experimental error. Therefore, most or all of the observed rotation is due to the rotation of the protein itself. These results indicate that *lin*-benzoadenosine is bound rigidly to the C subunit similar to *lin*-benzo-ADP.

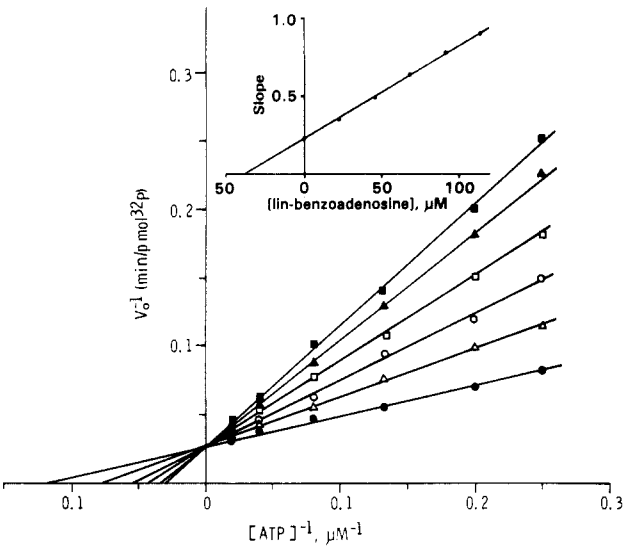

FIGURE 1: Competitive inhibition of catalytic subunit activity by *lin*-benzoadenosine. The catalytic subunit (10 ng) was incubated at 30 °C in a 0.2-mL volume containing 50 mM MOPS (pH 7.0), 10 mM $MgSO_4$, 0.25 mg/mL bovine serum albumin, 0.15 mM Ser-peptide, [γ-^{32}P]ATP concentrations from 4 to 50 μM (1200 cpm/pmol), and *lin*-benzoadenosine concentrations of 0 (●), 22.8 (△), 45.6 (○), 68.4 (□), 91.2 (▲), and 114 μM (■). (Inset) Slope replot of the data. The abscissa intercept corresponds to a K_i of 38 μM.

The K_d of 33.4 ± 2.5 μM of *lin*-benzoadenosine for the free catalytic subunit determined by an addition titration also agrees well with the kinetically determined K_i of 37.4 ± 4.1 μM. The K_d of *lin*-benzoadenosine determined here corresponds well with our previously determined K_d of 32 μM for adenosine (Bhatnagar et al., 1983) from fluorometric displacement titrations. The Scatchard analysis of the data obtained from the addition titration in this study showed that 1 mol of *lin*-benzoadenosine was bound per mole of the catalytic subunit.

Effect of Adenine Nucleotides on Inactivation of the Catalytic Subunit by Chemical Modification. The rates of inactivation of the catalytic subunit by FSBA, NBD-Cl, and DTNB in the presence of various nucleotides were examined. The inactivation reactions were carried out as previously described in the presence or absence of Mg^{2+}. Table I summarizes the ability of these nucleotides to protect the catalytic subunit from covalent modification by the chemical reagents. The data show that free ATP or ATP in the presence of equimolar concentrations of Mg^{2+} is ineffective in preventing inactivation of the enzyme. However, 2 mM ATP in the presence of excess magnesium chloride (10 mM) almost completely protected the C subunit from inactivation (Table

Table I: Effect of Adenine Nucleotides and Magnesium on Inactivation of the cAMP-Dependent Protein Kinase Catalytic Subunit by Chemical Reagents[a]

addition to reaction mixture	% residual activity: 1 mM FSBA	0.5 mM NBD-Cl	0.25 mM DTNB
control[b]	95	96	95
none	28	24	20
ATP (2 mM)	30	27	24
Mg^{2+} (10 mM)	36	31	30
Mg^{2+} (2 mM) + ATP (2 mM)	30	29	25
Mg^{2+} (10 mM) + ATP (10 mM)	38	42	36
Mg^{2+} (10 mM) + ATP (2 mM)	96	92	90
Ado (2 mM)	46	41	40
Mg^{2+} (10 mM) + Ado (2 mM)	48	45	49
AMP (2 mM)	32	22	19
AMP (10 mM)	44	38	35
Mg^{2+} (10 mM) + AMP (2 mM)	37	27	26

[a] The catalytic subunit (60 μg/mL) was incubated with the specified concentration of reagent in 0.20 M MOPS buffer (pH 7.0) as described under Materials and Methods. Mg^{2+} was added as the chloride salt. [b] Activity of 100% (in the absence of inhibitors, at time 0 min) was 16 μmol of ^{32}P incorporated·min^{-1}·(mg of catalytic subunit)$^{-1}$ with Ser-peptide as substrate.

I). AMP (2 mM) afforded no protection against inactivation. Adenosine (2 mM) slightly decreased the rate of inactivation, but it was much less effective than MgATP. These protection experiments provide evidence that FSBA, NBD-Cl, and DTNB inhibit the C subunit by reacting with the amino acid residues that effectively constitute the region complementary to the β- and γ-phosphates of the ATP molecule.

Binding of lin-Benzoadenine Nucleotides to the Chemically Modified Catalytic Subunit. FSBA, NBD-Cl, and DTNB have been used to inactivate the catalytic subunit. All three of these reagents have been postulated to modify residues at or near the nucleotide binding site of the catalytic subunit. To determine what effect these compounds had on nucleotide binding, the C subunit was reacted with the respective reagents as described under Materials and Methods and nucleotide binding to the modified enzyme was subsequently measured by fluorescence-polarization titrations.

The binding of *lin*-benzoadenosine to the chemically modified catalytic subunit is unaffected even when the enzyme is almost totally inactivated by either FSBA, NBD-Cl, or DTNB (Table II). We found almost identical binding affinities of *lin*-benzoadenosine for the catalytic subunit obtained from fluorescence-polarization addition titrations (described under Materials and Methods) performed on the control (unmodified) enzyme, the inactivated enzyme, or the enzyme protected from inactivation by the presence of 10 mM $MgCl_2$ and 2 mM ATP. The Scatchard analysis of the data obtained from these addition titrations also reveals that the K_d values were not derived in these experiments from any subpopulation of the catalytic subunit in the medium but from all the subunit molecules because the number of nucleotide binding sites in each case was approximately one site per C subunit. These results strongly suggest that the adenine and ribose binding regions of the ATP binding site on the catalytic subunit are not altered by chemical inactivation of the enzyme by FSBA, NBD-Cl, or DTNB.

We previously reported that FSBA, NBD-Cl, and ethoxyformic anhydride prevented *lin*-benzo-ADP binding to the modified enzyme (Hartl et al., 1983). We have further investigated *lin*-benzo-ATP binding to greater than 90% inactivated catalytic subunit following reaction with 1 mM FSBA, 0.5 mM NBD-Cl, or 0.25 mM DTNB. *lin*-Benzo-ATP binding to the modified enzymes was measured by fluorescence-polarization addition titrations. We found that FSBA, NBD-Cl, and DTNB modification of the catalytic subunit also prevents *lin*-benzo-ATP from binding to the inactivated catalytic subunit (data not shown).

Table II: Effect of Chemical Modification of the Catalytic Subunit on Binding of *lin*-Benzoadenosine[a]

addition to reaction mixture	% residual activity[b]	K_d (μM)[c]	$\bar{n}$[c]
5% Me_2SO (control)	98	28.1	1.06
Mg^{2+} (10 mM) + ATP (2 mM) + FSBA (1 mM)	91	33.2	0.98
FSBA (1 mM)	12	46.2	1.11
5% acetonitrile (control)	94	35.1	1.17
Mg^{2+} (10 mM) + ATP (2 mM) + NBD-Cl (0.5 mM)	89	36.4	1.14
NBD-Cl (0.5 mM)	15	34.6	1.12
control	94	25.8	1.09
Mg^{2+} (10 mM) + ATP (2 mM) + DTNB (0.25 mM)	88	27.3	1.08
DTNB (0.25 mM)	13	23.8	0.96

[a] The catalytic subunit (0.5–1 mg/mL) was incubated with the specified concentrations of reactants with or without metal nucleotide in 0.20 M MOPS (pH 7.0) at 30 °C as described under Materials and Methods. After 60 min of incubation the unreacted reagent and the catalytic subunit were separated as described. This enzyme was then used for polarization addition titration studies as described under Materials and Methods. [b] Percent residual activity refers to the catalytic activity remaining after the 60-min incubation of the catalytic subunit with the reagent. Activity of 100% was 15.6 μmol·min^{-1}·(mg of catalytic subunit)$^{-1}$ with Ser-peptide as substrate. [c] K_d values and the number of binding sites per enzyme ($\bar{n}$) were determined from Scatchard plots of data obtained from fluorescence-polarization addition titrations.

Incubation of the NBD-Cl-inactivated C subunit with excess 2-mercaptoethanol results in recovery of 85–90% of the original phosphotransferase activity (Hartl & Roskoski, 1982). *lin*-Benzo-ADP binding to the NBD-Cl-modified catalytic subunit was further investigated. *lin*-Benzo-ADP was used for most of our experiments instead of *lin*-benzo-ATP because the C subunit of the protein kinase possesses intrinsic adenosinetriphosphatase (ATPase) activity (Armstrong et al., 1979b) for which *lin*-benzo-ATP is a substrate (Hartl et al., 1983). The catalytic subunit was incubated for 60 min with 5% acetonitrile or treated with 0.5 mM NBD-Cl (in acetonitrile) in the presence or absence of Mg^{2+} (10 mM) and ATP (2 mM). Greater than 95% of the phosphotransferase activity is retained in the control. Less than 5% of the activity is present in the NBD-Cl-treated enzyme whereas MgATP was capable of affording greater than 85% protection against inactivation (Table III). Binding of *lin*-benzo-ADP to the control and treated enzyme was then measured by the fluorescence-polarization addition titration (described under Materials and Methods). The K_d of *lin*-benzo-ADP for the control catalytic subunit (incubation with acetonitrile followed by gel filtration) compares well with the K_d of 10.6 μM obtained for the untreated catalytic subunit from similar addition titrations (Hartl et al., 1983). In the case of enzyme modified with NBD-Cl, however, there is no increase in polarization observed following addition of *lin*-benzo-ADP and 10 mM $MgSO_4$, demonstrating no measurable binding of the nucleotide to the catalytic subunit under these conditions. Enzyme protected from inactivation by MgATP bound *lin*-benzo-ADP as well as did untreated catalytic subunit (K_d = 10.9 μM).

In order to eliminate the possibility of the lack of binding of the nucleotide to the catalytic subunit as a result of denaturation of enzyme during chemical modification, the modified enzyme was reactivated by incubation with 100 mM 2-mercaptoethanol (15 min). The polarization increased sig-

Table III: Binding of *lin*-Benzo-ADP to the NBD-Cl-Modified Catalytic Subunit[a]

addition to reaction mixture	phosphotransferase activity [μmol·min^{-1}·(mg of protein)$^{-1}$]	K_d of *lin*-benzo-ADP (μM)	$\bar{n}$[e]
5% acetonitrile	15.4[b]	9.8	1.06
Mg^{2+} (10 mM) + ADP (2 mM) + NBD-Cl (0.5 mM)	13.7[b]	10.9	0.97
NBD-Cl (0.5 mM)	0.7[b]	ND[d]	ND[d]
NBD-Cl (0.5 mM) + 2-mercaptoethanol (0.1 M)[c]	10.5	11.4	0.89

[a] The catalytic subunit (0.5–1 mg/mL) was incubated for 60 min with 0.5 mM NBD-Cl at 30 °C in 0.2 M MOPS (pH 7.0) as described under Materials and Methods. The unreacted NBD-Cl and the catalytic subunit were separated as described. This enzyme was then used for fluorescence-polarization addition titration studies to determine the dissociation constants of *lin*-benzo-ADP as described under Materials and Methods. [b] The catalytic activity refers to that remaining after the 60-min incubation. The phosphotransferase activity of the catalytic subunit before the incubation was 16 μmol·min^{-1}·(mg of catalytic subunit)$^{-1}$. [c] The NBD-Cl-inactivated C subunit was reactivated to 75% of the original phosphotransferase activity by incubation with excess 2-mercaptoethanol (100 mM) for 15–20 min as described by Hartl & Roskoski (1982). [d] ND, no detectable binding of the nucleotide to the catalytic subunit. [e] $\bar{n}$, number of binding sites per catalytic subunit.

nificantly with reactivation of enzyme, indicating binding of the nucleotide to the catalytic subunit. An addition titration performed on the reactivated enzyme gave a K_d for *lin*-benzo-ADP similar to that obtained for the control enzyme (Table III). Kinetic measurement of the activity of this enzyme indicated that the enzyme was partially reactivated. The reason for only partial reactivation is not known, but it agrees with our previous results (Hartl & Roskoski, 1982).

The dissociation constant (K_d) of AMP for the control and modified catalytic subunit was determined by displacing *lin*-benzoadenosine bound to the enzyme with increasing concentrations of the competing nucleotide, AMP, as described under Materials and Methods. The results for the binding of AMP to the catalytic subunit inactivated by 1 mM FSBA, 0.5 mM NBD-Cl, and 0.25 mM DTNB are summarized in Table IV. The K_d values of AMP for the unmodified enzyme in all three cases agree well with K_d = 642 μM determined by fluorescence (Bhatnagar et al., 1983) and K_i = 640 μM from steady-state enzyme kinetics for the free catalytic subunit (Cook et al., 1982). The enzyme used in these determinations retained 90% of its original activity over the time period (60 min) required for the specified concentrations (Table IV) of FSBA, NBD-Cl, or DTNB alone to inhibit the enzyme by greater than 90%. The covalently modified enzyme in all three cases bound AMP. This is demonstrated by the displacement of *lin*-benzoadenosine bound to the modified C subunit in each of the three cases by AMP (not shown). However, the K_d values of AMP for the modified kinase subunit are 25–30% higher than those for the respective control enzyme (Table IV). This indicates that there is a reduction in binding affinity of AMP for the catalytic subunit as a result of chemical modification of the enzyme by FSBA, NBD-Cl, and DTNB.

Similar fluorescence-polarization displacement titrations with ADP or ATP were performed on the modified (FSBA, NBD-Cl, and DTNB) and unmodified C subunit with *lin*-benzoadenosine as the fluorescent probe. ADP and ATP were capable of displacing *lin*-benzoadenosine bound to the unmodified enzyme, demonstrated by a decrease in polarization of the bound fluorescent ligand with increasing concentrations of either ADP or ATP. K_d values of 9.9, 10.4, and 9.6 μM for ADP for the control C subunit determined by displacement

Table IV: Effect of Chemical Modification of the Catalytic Subunit on AMP Binding[a]

addition to reaction mixture	% residual activity[b]	K_d (μM)[c]
5% Me_2SO	91	682
FSBA (1 mM)	11	818
5% acetonitrile	89	673
NBD-Cl (0.5 mM)	9	856
control	88	654
DTNB (0.25 mM)	8	804

[a] The catalytic subunit was inactivated by chemical modification as described in the legend for Table III. The covalently modified catalytic subunit, separated from excess reagent, was then used for fluorescence-polarization displacement titrations. [b] Percent residual activity refers to the catalytic activity remaining after 60-min incubation of the catalytic subunit with the chemical modifier. Activity of 100% was 15.6 μmol·min^{-1}·(mg of catalytic subunit)$^{-1}$. [c] K_d values of AMP for the catalytic subunit were determined by fluorescence-polarization displacement titrations as described under Materials and Methods.

titration using *lin*-benzoadenosine agree well with the previously determined K_d value for ADP of 10 μM (Bhatnagar et al., 1983) and the K_i value of 9 μM (Hartl et al., 1983) for the free catalytic subunit. Although ATP displaced *lin*-benzoadenosine bound to the unmodified C subunit, K_d values for ATP were not determined because the intrinsic ATPase activity of the C subunit generated ADP during the displacement titration. The cAMP-dependent protein kinase catalytic subunit possesses intrinsic ATPase activity (Armstrong et al., 1979b) for which *lin*-benzo-ADP is a substrate (Hartl et al., 1983). On the other hand, ADP and ATP concentrations of up to 1 mM were unable to displace *lin*-benzoadenosine bound to the catalytic subunit modified by either FSBA, NBD-Cl, or DTNB. These concentrations of ADP or ATP were more than sufficient to displace all *lin*-benzoadenosine bound to the unmodified catalytic subunit. Thus, FSBA, NBD-Cl, and DTNB, which are thought to modify residues that are at or near the active site of the catalytic subunit, appear to act in part by inhibiting nucleotide binding with respect to the triphosphate part of the ATP molecule.

Inactivation of the Catalytic Subunit by Covalent Chemical Modification in the Presence of Divalent Metal Ions. The effect of divalent metal ions on the inactivation of the catalytic subunit by FSBA, NBD-Cl, and DTNB was also investigated. The metal ions used in these experiments were those that promoted binding of nucleotide to the catalytic subunit (Bhatnagar et al., 1983). The metal ions at the selected concentrations (greater than 3 times K_{app}) by themselves did not afford significant protection against inactivation (Table V). Instead, it appears that the inactivating reaction is slightly accelerated in the presence of Cd^{2+}, Ca^{2+}, and Sr^{2+}.

Alkylation studies with a variety of reagents have indicated that at least one and possibly both cysteine residue(s) of the catalytic subunit (Shoji et al., 1983) is (are) situated near the active site of the enzyme (Bechtel et al., 1977; Peters et al., 1977; Sugden et al., 1976; Kupfer et al., 1979; Armstrong & Kaiser, 1978; Hartl & Roskoski, 1982; Nelson & Taylor, 1981). Our results (Hartl & Roskoski, 1982) have indicated that inactivation of the catalytic subunit by NBD-Cl, determined spectrophotometrically and radioisotopically, is associated with modification of 2.1 ± 0.15 mol of cysteine/mol of catalytic subunit. Activity can be restored by treating inactivated enzyme with 2-mercaptoethanol. MgATP protects one cysteine on the average from modification while protecting against inactivation. The results summarized in Table III demonstrate that nucleotide binding to the catalytic subunit is inhibited by modification of both cysteines (Cys-199 and

Table V: Metal Ions Fail To Protect against Inactivation of the Catalytic Subunit by Various Chemical Inhibitors[a]

addition to reaction mixture	% residual activity		
	1 mM FSBA	0.5 mM NBD-Cl	0.25 mM DTNB
control[b]	94	94	93
none	29	26	23
Mg^{2+} (10 mM)	36	32	28
Mn^{2+} (0.1 mM)	33	23	22
Co^{2+} (0.2 mM)	32	26	25
Cd^{2+} (10 mM)	18	14	16
Ca^{2+} (10 mM)	17	13	16
Sr^{2+} (18 mM)	19	16	17

[a] The catalytic subunit (60 μg/mL) was incubated with the specified concentration of the chemical reagent in 0.20 M MOPS buffer (pH 7.0) as described under Materials and Methods. Metal ions were added to the incubation mixture as their Cl^- salts. [b] Activity of 100% (original activity) was 16 μmol of ^{32}P incorporated·min^{-1}·(mg of catalytic subunit)$^{-1}$ with Ser-peptide as substrate.

Cys-343; Shoji et al., 1983) whereas there is no observed effect on nucleotide binding to the reactivated enzyme or the enzyme in which only one of the two cysteines is modified by NBD-Cl (i.e., in the presence of MgATP).

To determine if the protection afforded by MgATP (Table IV) is due to protection of a unique cysteine or whether this represents modification of an average of one cysteine per mole of catalytic subunit, the CNBr-generated fragments of the enzyme were examined. The catalytic subunit, which has been sequenced, contains seven methionine residues (Shoji et al., 1981). Thus cleavage with CNBr results in generation of eight fragments. The two cysteine residues (Cys-199 and Cys-343) are contained in the two largest fragments (CB7 and CB8, respectively; Shoji et al., 1981). The catalytic subunit was modified with [^{14}C]NBD-Cl in the absence and presence of 2 mM ATP and 10 mM Mg^{2+}. The modified enzyme was separated from excess NBD-Cl and digested with CNBr as described in the legend for Figure 2. The digests were subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Figure 2). The gels were sliced and solubilized with 30% H_2O_2, and their radioactivity was determined by liquid scintillation spectrometry. Figure 3 shows that the patterns of radioactivity are the same for enzyme modified in the absence or presence of MgATP. However, the total radioactivity in the peaks corresponding to CB7 and CB8 is decreased in the catalytic subunit modified in the presence of MgATP (Figure 3B). This indicates that MgATP protects against NBD-Cl inactivation by partially protecting both Cys-199 and Cys-343.

Discussion

lin-Benzoadenine nucleotides, which are stretched out by 2.4 Å in their adenine moiety (Scopes et al., 1977), bind as well to the nucleotide binding site of the catalytic subunit as do native nucleotides. This can be concluded from our observations as follows. First, *lin*-benzo-ADP (Hartl et al., 1983) and *lin*-benzoadenosine (Figure 1) are linear competitive inhibitors with respect to MgATP as are ADP and adenosine (Cook et al., 1982). Second, the K_i determined for *lin*-benzo-ADP (Hartl et al., 1983) and *lin*-benzoadenosine (see Results) from inhibition studies and the K_d determined for these from fluorescence-polarization studies are almost identical with the kinetically determined K_i values of ADP and adenosine, respectively. In addition, *lin*-benzo-ATP is a good substrate for the catalytic subunit (Hartl et al., 1983). The polarization data (see Results) also indicate that *lin*-benzoadenosine is rigidly bound by the catalytic subunit. This is supported by nuclear magnetic resonance studies of Granot et al. (1979), who showed a strong interaction between the protein kinase and the adenosine portion of $Co(NH_3)_4ATP$. These fluorescent nucleotides, namely, *lin*-benzo-ATP, *lin*-benzo-ADP, and *lin*-benzoadenosine, provide another approach for studying nucleotide binding at the active site of the catalytic subunit.

The function of the enzyme regions that react with either FSBA or DTNB and NBD-Cl may be elucidated by considering the pattern of protection against modification afforded by the substrates. Both NBD-Cl and DTNB alkylate the two cysteines (Cys-199 and Cys-343; Shoji et al., 1983) of the catalytic subunit (Armstrong & Kaiser, 1978; Hartl & Ros-

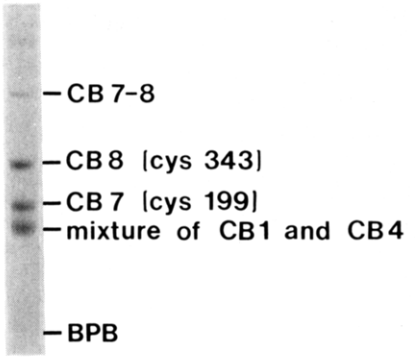

FIGURE 2: Polyacrylamide gel electrophoresis of CNBr fragments from the catalytic subunit. The catalytic subunit (0.1 mg) was modified with [^{14}C]NBD-Cl and separated from excess reagent on a 1 × 14 cm Sephadex G-50 column as described under Materials and Methods. [^{14}C]NBD incorporation into the catalytic subunit was determined as described in Hartl & Roskoski (1982). Excess unlabeled NBD-Cl was added, and the sample was dialyzed exhaustively against 5 mM MOPS (pH 7.0). After lyophilization, the enzyme was dissolved in 0.1 mL of 1% (w/v) CNBr in 72% formic acid. The digestion was carried out for 16 h at room temperature in the dark. After the addition of 1 mL of H_2O the sample was lyophilized. This was repeated twice with 0.5- and 0.25-mL volumes of H_2O. The sample was taken up in 0.1 mL of 10 mM sodium phosphate (pH 7.0) containing 1% SDS and boiled for 5 min. The digests (10–20 μg) were electrophoresed on 10% polyacrylamide gels in a sodium phosphate buffer system (Weber & Osborn, 1969) at 8 mA per gel, stained with Coomassie blue R250, and destained in acetic acid/ethanol/water (1/2.5/6.5). The two cysteines (at positions 199 and 343) are present on the two largest CNBr fragments, CB7 (residues 128–230) and CB8 (residues 231–349), respectively. The nomenclature for CNBr fragments is from Shoji et al. (1981).

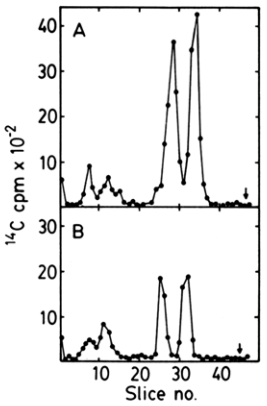

FIGURE 3: Analysis of [^{14}C]NBD-labeled CNBr fragments of catalytic subunit modified in the presence and absence of MgATP. The catalytic subunit was modified with [^{14}C]NBD-Cl in the absence (panel A) and presence (panel B) of 2 mM MgATP, digested with CNBr, and subjected to SDS–polyacrylamide gel electrophoresis as described in the legend to Figure 2. The gels were cut into 2-mm slices and solubilized overnight at 60 °C in 0.5 mL of 30% H_2O_2 in 7-mL scintillation vials. Budget-Solve (5 mL) was added, and the radioactivity was measured by liquid scintillation spectrometry.

koski, 1982) whereas FSBA covalently modifies Lys-71 concomitant with the loss of catalytic activity (Zoller et al., 1981). In the absence of Mg^{2+}, ATP (2 mM) does not protect against inactivation (Table I). In the presence of 10 mM Mg^{2+}, however, 2 mM ATP protects. AMP and adenosine, on the other hand, do not protect against inhibition or partially protect in the presence or absence of 10 mM Mg^{2+}. The divalent metal ion (Mg^{2+}) is required for the binding of the nucleotide to the enzyme as determined by fluorescence polarization (Bhatnagar et al., 1983). It has also been demonstrated earlier that ADP in the presence of Mg^{2+} protects the catalytic subunit from modification by NBD-Cl (Hartl & Roskoski, 1982). MgADP as well as MgAMP-PNP fully protected the enzyme from inactivation by FSBA (Hixson & Krebs, 1979). Protein or peptide substrates do not prevent C subunit inactivation by either FSBA (Hixson & Krebs, 1979) or NBD-Cl (Hartl & Roskoski, 1982). Divalent metal ions that promote binding of the nucleotide to the catalytic subunit (Bhatnagar et al., 1983) did not offer significant protection from chemical modification inactivation by FSBA, NBD-Cl, or DTNB (Table II). The metal ions, in the absence of nucleotide, may only weakly occupy the inhibitory site on the catalytic subunit at these concentrations (Armstrong et al., 1979a; Granot et al., 1980). ATP (2 mM) in the presence of equimolar concentrations of Mg^{2+} does not protect against covalent modification by either of the three reagents (Table V). Under these conditions, metal ions will be chelated by ATP and very little metal ion ($[Mg^{2+}]_{free}$ = 0.03 mM; Cook et al., 1982) would be available to bind nucleotide to the inhibitory site on the C subunit. These protection experiments constitute one line of evidence consistent with the notion that lysine-71 and cysteines 199 and -343 are present near or affect the active site region on the C subunit complementary to the β- and γ-phosphates of the ATP molecule.

The specific effects of covalent modification of lysine-71 and cysteines-199 and -343 on nucleotide binding were further characterized by fluorescence-polarization titrations with *lin*-benzoadenine nucleotides. Modification of these residues resulting in the inactivation of phosphotransferase activity did not alter the binding of *lin*-benzoadenosine to the enzyme (Table II). *lin*-Benzo-ADP and *lin*-benzo-ATP, however, failed to bind to the inactivated C subunit in the presence of 10 mM $MgSO_4$. Binding of AMP to such enzyme was determined indirectly by displacement of *lin*-benzoadenosine. Although AMP did bind to the C subunit modified at lysine-71 or cysteines-199 and -343 by FSBA or NBD-Cl and DTNB, respectively, there was a greater than 25% reduction in affinity of the catalytic subunit for AMP (Table IV). Fluorescence-polarization displacement titrations with ADP and ATP indicate that neither displaced *lin*-benzoadenosine bound to the covalently modified enzyme. This provides additional evidence that ADP and ATP cannot bind to the chemically modified catalytic subunit. These fluorescence-polarization binding studies suggest that modification of either lysine-71 or cysteines-199 and -343 of the C subunit does not affect the adenine or the ribose binding regions of the active site of the enzyme but does alter the triphosphate binding region on the catalytic subunit. This effect is much more discernible in the region on the C subunit complementary to the β- and γ-phosphates of the ATP molecule as compared to the region complementary to the α-phosphate of the ATP molecule in the nucleotide binding portion of the C subunit.

Our results (Figures 2 and 3) indicate that both cysteines of the catalytic subunit (Cys-199 and Cys-343) are covalently modified with NBD-Cl or DTNB concomitant with inactivation of the catalytic subunit. MgATP, however, protects against inhibition by partially protecting both cysteine residues. Recently Nelson & Taylor (1983) have reported that alkylation of the dissociated catalytic subunit with iodoacetic acid resulted in loss of activity that was associated with the alkylation of both cysteine residues. When C subunit activity was protected from inhibition (in the holoenzyme), Cys-343 was fully alkylated and Cys-199 was completely protected from chemical modification. Their results have established that Cys-343 is not essential for enzymatic activity and, furthermore, indicate that interaction of the C and R subunits in the holoenzyme selectively protects Cys-199. Our inability to differentially label Cys-199 [as compared to that of Nelson & Taylor (1983)] could be due to differences in the mechanism of protection by the R subunit and MgATP. Nelson & Taylor (1981) found that in the presence of MgATP both Cys-199 and Cys-343 are protected against modification by 70 mM iodoacetate, whereas we find that MgATP on the average reduces the incorporation of [^{14}C]NBD in the C subunit by 50% (from 2 mol to 1 mol/mol of C subunit) while retaining 80% of the phosphotransferase activity (Hartl & Roskoski, 1982). These dissimilarities could be due to the differences in the reactivity of the reagents used in the two studies.

In summary, three residues (lysine-71, cysteine-199, and cysteine-343), far apart in the polypeptide chain, effectively constitute the region on the catalytic subunit that interacts with the β- and γ-phosphates of ATP. Even though both cysteines are possibly in close proximity to the ATP binding site, Cys-343 alone does not seem to be essential for catalysis. However, lysine-71 and cysteines-199 and -343 are spaced far enough apart in the active site such that modification of the lysine by FSBA does not inhibit the cysteines from further reaction. This is demonstrated by our results (Hartl & Roskoski, 1982), which show that pretreatment of the enzyme with FSBA fails to alter the stoichiometry or rate of the NBD modification of the C subunit. The inactivation of C subunit activity by NBD-Cl, FSBA, and DTNB is due, at least in part, to the failure of the modified enzyme to bind the metal–nucleoside triphosphate substrate. The chemically modified enzymes bind nucleosides (adenosine, *lin*-benzoadenosine) with K_d's and stoichiometries identical with those of the unmodified enzyme. Following modification with FSBA, the adenine–ribose portion of the molecule must leave that region of the active site to permit binding of *lin*-benzoadenosine or adenosine.

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References

Armstrong, R. N., & Kaiser, E. T. (1978) *Biochemistry 17*, 2840.

Armstrong, R. N., Kondo, H., Granot, J., Kaiser, E. T., & Mildvan, A. S. (1979a) *Biochemistry 18*, 1230.

Armstrong, R. N., Kondo, H., & Kaiser, E. T. (1979b) *Proc. Natl. Acad. Sci. U.S.A. 76*, 722.

Bechtel, P. J., Beavo, J. A., & Krebs, E. G. (1977) *J. Biol. Chem. 252*, 2691.

Bhatnagar, D., Roskoski, R., Jr., Rosendahl, M. S., & Leonard, N. J. (1983) *Biochemistry 22*, 6310.

Bradford, H. F. (1976) *Anal. Biochem. 72*, 248.

Cook, P. F., Neville, M. E., Vrana, K. E., Hartl, F. T., & Roskoski, R., Jr. (1982) *Biochemistry 21*, 5794.

Granot, J., Kondo, H., Armstrong, R. N., Mildvan, A. S., & Kaiser, E. T. (1979) *Biochemistry 18*, 2339.
Granot, J., Mildvan, A. S., Hyama, K., Kondo, H., & Kaiser, E. T. (1980) *J. Biol. Chem. 255*, 4569.
Hartl, F. T., & Roskoski, R., Jr. (1982) *Biochemistry 21*, 5175.
Hartl, F. T., Roskoski, R., Jr., Rosendahl, M. S., & Leonard, N. J. (1983) *Biochemistry 22*, 2347.
Hixson, C. S., & Krebs, E. G. (1979) *J. Biol. Chem. 254*, 7509.
Hoppe, J., Marutzky, R., Freist, W., & Wagner, K. G. (1977) *Eur. J. Biochem. 80*, 369.
Hoppe, J., Freist, W., Marutzky, R., & Shaltiel, S. (1978) *Eur. J. Biochem. 90*, 427.
Kupfer, A., Gani, V., Jiminez, J., & Shaltiel, S. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3073.
Leonard, N. J., Sprecker, M. A., & Morrice, A. G. (1976) *J. Am. Chem. Soc. 98*, 3987.
Leonard, N. J., Scopes, D. I. C., Van Der Lijn, P., & Barrio, J. R. (1978) *Biochemistry 17*, 3677.
Lowry, D. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.
Nelson, N. C., & Taylor, S. S. (1981) *J. Biol. Chem. 256*, 3743.
Nelson, N. C., & Taylor, S. S. (1983) *J. Biol. Chem. 258*, 10981.
Perrin, F. (1926) *J. Phys. Radium 7*, 390.
Perrin, F. (1929) *Ann. Phys. (Paris) 12*, 169.
Peters, K. A., Demaille, J. G., & Fischer, E. H. (1977) *Biochemistry 16*, 5691.
Rosevear, P. R., Bramson, H. N., O'Brian, C., Kaiser, E. T., & Mildvan, A. S. (1983) *Biochemistry 22*, 3439.
Roskoski, R., Jr. (1983) *Methods Enzymol. 99*, 3.
Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660.
Schmidt, M. J., Truex, L. L., Leonard, N. J., Scopes, D. I. C., & Barrio, J. R. (1978) *J. Cyclic Nucleotide Res. 4*, 201.
Scopes, D. I. C., Barrio, J. R., & Leonard, N. J. (1977) *Science (Washington, D.C.) 195*, 296.
Shoji, S., Parmelee, D. C., Wade, R. D., Kumar, S., Ericsson, L. H., Walsh, K. A., Neurath, H., Long, G. L., Demaille, J. C., Fischer, E. H., & Titani, K. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 848.
Shoji, S., Ericsson, L. H., Walsh, K. A., Fischer, E. H., & Titani, K. (1983) *Biochemistry 22*, 3702.
Sugden, P. H., Holladay, L. A., Reimann, E. M., & Corbin, J. D. (1976) *Biochem. J. 159*, 409.
Takio, K., Smith, S. B., Krebs, E. G., Walsh, K. A., & Titani, K. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2544.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406.
Witt, J. J., & Roskoski, R., Jr. (1975) *Biochemistry 14*, 4503.
Zoller, M. J., & Taylor, S. S. (1979) *J. Biol. Chem. 254*, 8363.
Zoller, M. J., Nelson, N. C., & Taylor, S. S. (1981) *J. Biol. Chem. 256*, 10837.

5′-[*p*-(Fluorosulfonyl)benzoyl]adenosine-Mediated Inactivation of *S*-Adenosylhomocysteinase†

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ABSTRACT: Rat liver *S*-adenosylhomocysteinase (EC 3.3.1.1) is inactivated by 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine following pseudo-first-order kinetics. A plot of the apparent first-order rate constant for inactivation vs. the 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine concentration exhibits a hyperbolic curve indicative of the formation of a reversible enzyme–reagent complex prior to the inactivation. Values of 71.0 ± 7.7 μM and 0.14 ± 0.01 min^{-1} are estimated for K_d and k, respectively, at pH 8.25 and 25 °C. The substrate adenosine and a competitive inhibitor adenine completely protect the enzyme against inactivation. Values of dissociation constant for these ligands calculated from the protection experiments agree well with those obtained by other means, indicating that 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine competes with these ligands for the same binding site. The inactivation is not reversed by dialysis against phosphate buffer or tris(hydroxymethyl)aminomethane hydrochloride buffer, but a full enzyme activity is regained by treatment with dithiothreitol. The inactivation is not accompanied by covalent attachment of the reagent but is correlated with the loss of two sulfhydryl groups per enzyme subunit. Thus, the inactivation appears to result from a reagent-mediated formation of a disulfide between two cysteine residues in close proximity. The 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine-modified enzyme which is not capable of catalyzing the overall reaction can still catalyze the partial reactions such as the oxidation of the 3′-hydroxyl and the abstraction of the 4′-proton of adenosine.

S-Adenosylhomocysteinase (EC 3.3.1.1), which catalyzes the reversible hydrolysis of *S*-adenosylhomocysteine to adenosine and homocysteine, has been purified to apparent homogeneity from a variety of sources. The enzymes from mammalian sources are tetramers consisting of subunits with molecular weights of 45 000–48 000 (Schatz et al., 1979; Palmer & Abeles, 1979; Fujioka & Takata, 1981; Døskeland & Ueland, 1982) and, as far as examined, contain 4 mol of tightly bound NAD per mol of enzyme (Richards et al., 1978; Palmer & Abeles, 1979; Fujioka & Takata, 1981; Ueland, 1982a). The enzyme-bound NAD is essential for catalysis, and a mechanism of action of *S*-adenosylhomocysteinase is proposed that involves the oxidation/reduction of substrates by the coenzyme in the catalytic cycle (Palmer & Abeles, 1976, 1979).

Amino acid residues involved in the catalytic activity of rat liver *S*-adenosylhomocysteinase have been investigated in this laboratory. A histidine (Gomi & Fujioka, 1983), arginine

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