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Bovine Brain Adenosine 3′,5′-Monophosphate Dependent Protein Kinase. Mechanism of Regulatory Subunit Inhibition of the Catalytic Subunit†

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ABSTRACT: Adenosine 3′,5′-monophosphate (cAMP) dependent protein kinase (EC 2.7.1.37) catalyzes the phosphorylation of serine and threonine residues of a number of proteins according to the following chemical equation: ATP + protein → phosphoprotein + ADP. The DEAE-cellulose peak II holoenzyme from bovine brain, which is composed of regulatory and catalytic subunits, is resistant to ethoxyformic anhydride inactivation. After adding cAMP, the protein kinase becomes susceptible to ethoxyformic anhydride inhibition. Ethoxyformic anhydride (2 m*M*) inhibits the enzyme 50% (5 min, pH 6.5, 30°) in the presence of 10 μM cAMP, but less than 5% in its absence. The substrate, Mg^{2+}-ATP, protects against inactivation suggesting that inhibition is associated with modification of the active site. Addition of regulatory subunit or Mg^{2+}-ATP to the isolated catalytic subunit also prevents ethoxyformic anhydride inactivation. These results suggest that the regulatory subunit shields the active site of the catalytic subunit thereby inhibiting it. In contrast to the bovine brain or muscle DEAE-cellulose peak II holoenzyme, the bovine muscle peak I holoenzyme is susceptible to ethoxyformic anhydride inactivation in the absence of cAMP.

Protein kinase (EC 2.7.1.37) catalyzes the phosphorylation of polypeptidic serine and threonine hydroxyl groups according to the chemical equation: ATP + protein → phosphoprotein + ADP. Through phosphorylation, protein kinases regulate the activity of many enzymes including phosphorylase kinase (Walsh et al., 1968), glycogen synthetase (Soderling et al., 1970), and hormone-sensitive lipase (Huttunen et al., 1970). They also appear to be involved in membrane permeability (Johnson et al., 1972), chromosome function (Langan, 1968), protein synthesis (Eil and Wool, 1973), and steroidogenesis (Walton et al., 1971).

A major class of protein kinase requires adenosine 3′,5′-cyclic monophosphate (cAMP)[1] for activity. The mechanism of activation is expressed by the equation RC +

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[1] Abbreviations used are: cAMP, adenosine 3′,5′-monophosphate; Mops, morpholinopropanesulfonic acid.

Table I: Ethoxyformic Anhydride Inhibition of Protein Kinase.[a]

Preincubation	Enzyme Assay [cAMP] (μM)	Activity (pmol/min)	[cAMP] (μM)	Activity (pmol/min)
1. Control	0	15	10	131
2. Ethoxyformic anhydride (2.0 mM)	0	13	10	119
3. cAMP (10 μM)	5	113	15	107
4. cAMP (10 μM), ethoxyformic anhydride (2.0 mM)	5	60	15	58

[a] To give the final specified concentration, aliquots (2 μl of ethoxyformic anhydride (100 mM) in acetonitrile) were added to 100 μl of enzyme extract (0.5 mg of protein/ml) in 20 mM Mops (pH 6.7). After a 5-min preincubation at 30°, aliquots (25 μl) were assayed for protein kinase activity in a final volume of 50 μl at 30° as previously described (Witt and Roskoski, 1975b). Ethoxyformic anhydride was dissolved in acetonitrile immediately before use. Acetonitrile alone was included with the controls.

Table II: Effect of Ethoxyformic Anhydride on Resolved and Combined Regulatory and Catalytic Subunit Fractions.[a]

Fraction	Enzyme Activity (pmol/min) −cAMP	+cAMP
1. Catalytic subunit		
Control	355	435
Ethoxyformic anhydride	60	162
2. Catalytic and regulatory subunits		
Control	35	345
Ethoxyformic anhydride	41	320
3. Regulatory subunit		
Control	9	92.5
Ethoxyformic anhydride	2	45

[a] The regulatory and catalytic fractions were prepared as described in the Experimental Section. Ethoxyformic anhydride treatment of the regulatory (30 μg of protein) and catalytic (20 μg) fractions and enzyme assays were performed as described in Table I. The regulatory and catalytic subunit fractions were incubated 2 min (0°) prior to addition of ethoxyformic anhydride.

cAMP $\rightleftharpoons$ C + R·cAMP, in which RC represents the holoenzyme and R and C are the regulatory and catalytic subunits, respectively (Gill and Garren, 1970; Tao et al., 1970; Kumon et al., 1970; Reimann and coworkers, 1971). In holoenzyme, the regulatory subunit inhibits the catalytic subunit. cAMP promotes the dissociation of the RC complex producing a free catalytic subunit and a cAMP-regulatory subunit complex. The freed catalytic subunit is active and cAMP independent. Combining isolated C + R·cAMP subunits results in the release of cAMP and the regeneration of a cAMP-dependent holoenzyme (Brostrom et al., 1971).

Although the mechanism of cAMP activation of the holoenzyme is well documented (cf. Walsh and Krebs, 1973), the means whereby the regulatory subunit inactivates the catalytic subunit has not been established. Of the two major fractions resolved by DEAE-cellulose chromatography (protein kinase I and II), kinase II is the major fraction in brain (Corbin et al., 1975). Based on results obtained by chemical modification of bovine brain kinase II and the conditions for binding to a nucleotide analog affinity resin, we propose that the regulatory subunit shields the active site of the catalytic subunit thereby inactivating it.

Experimental Section

Materials. Ethoxyformic anhydride, Blue Dextran, and Sepharose 4-B were purchased from Sigma Chemical Co. Blue Dextran-Sepharose was prepared by the procedure of Ryan and Vestling (1974).

Resolution of Catalytic and Regulatory Subunits. Bovine brain protein kinase holoenzyme was prepared by DEAE-cellulose chromatography (kinase II) and Sephadex gel filtration as previously described (Witt and Roskoski, 1975b). To resolve the regulatory from the catalytic subunit, enzyme extract (10 mg of protein in 2 ml) was incubated with 0.1 mM cAMP (10 min; 0° for this and subsequent procedures unless otherwise specified) and applied to a Blue Dextran-Sepharose column (0.9 × 3 cm) equilibrated with buffer A [4 mM Mops (pH 6.7), 0.5 mM dithiothreitol, 5 mM $MgCl_2$, and 5% glycerol (v/v)] to which 0.1 mM cAMP was added. Buffer A (3 × 1.5 ml) was used to elute the regulatory subunit fraction and 0.2 M KCl (3 × 1.5 ml) in buffer A was used to elute the catalytic subunit fraction. The respective fractions were combined and concentrated by pressure dialysis. To remove free cAMP, the fractions were dialyzed 14 hr against 200 vol of buffer A. These fractions were stored at 4° and were stable for at least 1 week.

The protein kinase assay using phosphocellulose paper absorption was performed as previously described (Witt and Roskoski, 1975b) with the following modifications: the phosphocellulose strips were quenched and rinsed in distilled water because seasonal variations in tap water produced variable blank values; additionally, the liquid scintillation vials and fluor were reused since radiolabel remains bound to the paper. Other methods and the sources of other materials are previously documented (Witt and Roskoski, 1975b).

Results

Ethoxyformic Anhydride Inhibition of Protein Kinase. After preincubating ethoxyformic anhydride with protein kinase in the absence of cAMP, enzyme activity (+ cAMP) is 95% that of the control (Table I). When the enzyme is preincubated with cAMP, however, ethoxyformic anhydride inhibits activity 50%. cAMP thus converts the enzyme from a resistant to a susceptible form. The amount of inhibition (in the absence of added cAMP) correlates with cAMP-independent enzyme activity. During storage (6 weeks, 4°), for example, cAMP independent protein kinase activity increases 3.5-fold. Ethoxyformic anhydride then inhibits stored enzyme 35% in the absence of cAMP. In order to minimize cAMP-independent activity, only enzyme stored less than 3 weeks (4°) was used in this study.

The inactivation of protein kinase in the presence of cAMP is proportional to ethoxyformic anhydride concentration and exhibits good pseudo-first-order kinetics (three half-times) during the 5-min reaction. Since the inhibitor hydrolyses with a $t_{1/2}$ of 27 min (pH 7) (Melchior and Fahrney, 1970), these kinetics do not hold during long incubations.

Ethoxyformic Anhydride Inactivation of the Catalytic Subunit Fraction. To gain further insight into the nature of the form of protein kinase susceptible to inactivation, studies on the resolved catalytic and regulatory subunit fractions were performed. The catalytic subunit fraction is stimulated only 20% by cAMP, while the recombined regulatory

Table III: Effect of Substrates on Ethoxyformic Anhydride Inactivation of the Catalytic Subunit Fraction.[a]

Addition	Enzyme Activity (% Control)
None	11
ATP (1 m*M*), $MgCl_2$ (5 m*M*)	92
ATP (1 m*M*)	20
$MgCl_2$ (5 m*M*)	11
Histone (2 mg/ml)	10
GTP (1 m*M*), $MgCl_2$ (5 m*M*)	11
ADP (1 m*M*), $MgCl_2$ (5 m*M*)	45
cAMP (1 m*M*), $MgCl_2$ (5 m*M*)	14

[a] Ethoxyformic anhydride treatment of the catalytic subunit fraction (30 μg) containing the designated substances at the specified concentrations and enzyme assays (−cAMP) were carried out as described in Table I. The catalytic subunit fraction was prepared as described in the Experimental Section.

Table IV: Characteristics of Protein Kinase Binding to Blue Dextran-Sepharose.[a]

	Enzyme Activity (pmol/min)	cAMP Binding (pmol/mg of Protein)
	−cAMP	
Eluent		
Buffer A	80	70
KCl (0.2 *M*)	5	2
	+cAMP	
Eluent		
Buffer A	18	67
KCl (0.2 *M*)	86	5.4

[a] Enzyme extract (10 mg of protein) was applied to Blue Dextran-Sepharose by the methodology described in the Experimental Section in the presence or absence of cAMP in the enzyme extract and eluting solutions, as specified. Enzyme activity was measured in the presence of 10 μ*M* cAMP (Witt and Roskoski, 1975b) and cAMP binding by the procedure of Gill and Garren (1970).

Table V: Binding of Catalytic Subunit to Blue Dextran-Sepharose.[a]

Components Added to Catalytic Subunit	Eluent (Enzyme Activity, pmol/min)	
	Buffer A	0.2 *M* KCl
None	20	400
1 m*M* ATP, 5 m*M* $MgCl_2$	380	30
Regulatory subunit[b]	410	15
Regulatory subunit[b] 10 μ*M* cAMP	35	380

[a] Catalytic subunit was prepared according to the methodology described in the Experimental Section. Catalytic subunit (250 μg of protein in 500 μl of buffer A) was applied to a Blue Dextran-Sepharose column (0.8 × 1.5 cm) in the presence of the specified components. Aliquots of buffer A (2 × 2 ml) and then 0.2 *M* KCl (2 × 2 ml) were passed through the column. Enzyme activity in the eluent was determined in the presence of 10 μ*M* cAMP (Witt and Roskoski, 1975b). [b] Regulatory and catalytic subunits were recombined as described in Table II prior to application to the column.

and catalytic fractions are stimulated 885% (Table II) indicating substantial resolution. Ethoxyformic anhydride treatment in the absence of cAMP inactivates the resolved catalytic subunit fraction 65–85% (Table II); it inhibits the recombined regulatory and catalytic fractions less than 10%. In agreement with inhibition studies of holoenzyme (± cAMP) outlined in Table I, these experiments suggest that ethoxyformic anhydride inhibits the catalytic subunit, but fails to inhibit the holoenzyme (combined regulatory and catalytic subunits). The regulatory subunit fraction thus protects the catalytic subunit from inactivation.

Effect of Substrates on Inhibition of the Catalytic Subunit Fraction. To learn more about the properties of inhibition, chemical modification was carried out in the presence of substrates and analogs. Mg^{2+}-ATP substantially protects the catalytic subunit fraction from ethoxyformic anhydride inactivation (Table III). ATP or Mg^{2+} alone fail to protect. With 5 m*M* $MgCl_2$, ATP concentrations of 1.0, 0.5, and 0.1 m*M* protect 85, 76, and 36%, respectively (not shown). Mg^{2+}-ADP partially protects the enzyme against inactivation. Adenosine and dATP produced modest protection in the presence of 5 m*M* $MgCl_2$. Adenine, 5′-AMP, pyrophosphate, GTP, ITP, UTP, and CTP, however, fail to protect. Even though cAMP is a competitive inhibitor of bovine brain protein kinase with ATP as variable substrate (Donnelly and coworkers, 1973), we are unable to demonstrate protection against ethoxyformic anhydride inactivation using 1 m*M* cAMP (5 m*M* $MgCl_2$). Preincubation of the enzyme with histone (1 mg/ml), casein (2 mg/ml), phosvitin (2 mg/ml), or protamine (2 mg/ml) fails to protect the catalytic subunit against inactivation. Contrariwise, histone accelerates the rate of inhibition when measured at earlier times (not shown).

The Mg^{2+}-ATP protection against inactivation provides presumptive evidence that inhibition is associated with chemical modification of an active-site residue. The regulatory subunit also protects the enzyme against inactivation (Table II). We therefore propose that the regulatory subunit shields—either physically or functionally—the Mg^{2+}-ATP site of the catalytic subunit. Studies performed with a Blue Dextran-Sepharose affinity resin support this scheme.

Affinity Chromatography of Protein Kinase. Blue Dextran-Sepharose affinity chromatography is useful in purifying a number of enzymes with nucleotide substrates (Thompson et al., 1975). Reactive blue 2 (Cibachron 3-GA), the chromophore of Blue Dextran, is a structural analog of the adenosine diphosphoryl portion of the nucleotide. Since this chromophore is an analog of ATP and ADP, a substrate and product of the enzyme reaction, experiments were performed to test its usefulness. In the absence of cAMP, protein kinase holoenzyme fails to bind to Blue Dextran-Sepharose; enzyme activity and cAMP-binding protein (regulatory subunit) elute with the low ionic strength charging buffer (Table IV). After adding cAMP, the regulatory subunit elutes, and the catalytic subunit activity binds. KCl (0.2 *M*) then elutes the enzyme activity.

That the ATP site of the catalytic subunit binds to the affinity ligand is supported by the following: ATP (1 m*M*)–$MgCl_2$ (5 m*M*) elutes 95% of the enzyme (not shown); preincubation of the catalytic subunit with Mg^{2+}-ATP prevents activity from binding to the resin (Table V). Although preincubation of the regulatory and catalytic subunit fractions prevents binding (Table V), the regulatory subunit fraction fails to elute the bound enzyme activity (not shown). That the native or recombined holoenzyme does not bind to the resin supports the idea that the ATP site is shielded. Binding of the free catalytic subunit indicates that the ATP site is accessible.

Determination of the pK_a of the Reactive Residue. Ethoxyformic anhydride inactivation of either the catalytic

subunit or enzyme in the presence of cAMP is dependent upon pH (Table VI). The apparent first-order rate constants (K_{app}) were calculated using the methodology of Burstein et al. (1974). A plot of K_{app} $[H^+]$ vs. K_{app} (values from Table VI) gives a straight line. A pK_a of 7.8 ± 0.3 and K_{max} of 0.15 ± 0.03 min^{-1} were calculated from the slope and ordinate intercept, respectively.

Reversal of Ethoxyformyl Inactivation. Attempts were made to reverse the inactivation of the catalytic subunit with hydroxylamine. Treatment of the inhibited enzyme with 20 m*M*, 200 m*M*, and 500 m*M* hydroxylamine for 10 min at 30° at neutral pH, followed by dialysis for 3 hr to remove the hydroxylamine, reactivated the enzyme 0, 25, and 35%, respectively. Hydroxylamine treatment for more than 10 min is unfeasible because of enzyme instability. Storage of inactivated enzyme for several days at 4° also fails to reactivate enzyme activity.

Ethoxyformic Anhydride Inactivation of Bovine Muscle Protein Kinase I and II. Tissues differ in the proportion of protein kinase activity which elutes from DEAE-cellulose at low ionic strength (protein kinase I) and high ionic strength (protein kinase II). The chief form of the brain enzyme, for example, is protein kinase II (Corbin et al., 1975). Skeletal muscle, however, contains appreciable amounts of protein kinase I and II activity. Experiments were carried out to test for ethoxyformic anhydride inhibition of protein kinase activity from muscle. Skeletal muscle protein kinase II holoenzyme is more resistant to ethoxyformic anhydride inactivation than protein kinase I. Ethoxyformic anhydride inhibits the former 25% under conditions where the latter is inhibited 72% (Table VII). Protein kinase II and I exhibit 8 and 1% cAMP-independent activity, respectively. This may account for a portion of the ethoxyformic anhydride inactivation of the protein kinase II observed in the absence of cAMP (vide ante). The substrate (1 m*M* ATP–5 m*M* $MgCl_2$) also protects protein kinase I from ethoxyformic anhydride inhibition. Like the brain enzyme, muscle protein kinase II holoenzyme fails to bind to the Blue Dextran-Sepharose affinity resin. Muscle kinase I, however, binds to the Blue Dextran-Sepharose (not shown). Ethoxyformic anhydride inhibition and Blue Dextran-Sepharose affinity chromatographic experiments support the contention that the active site of protein kinase II from muscle and brain holoenzymes is shielded. The Mg^{2+}-ATP site of kinase I, on the other hand, is accessible. The interaction of regulatory and catalytic subunits from kinase I thus differs from that of kinase II.

Table VI: pH Dependence of Ethoxyformic Anhydride Inhibition of Protein Kinase in the Presence of cAMP.[a]

pH	K_{app} (min^{-1})
7.1	0.023
7.5	0.046
8.0	0.091
8.5	0.120

[a] Enzyme (50 μg in 100 μl of 5 m*M* tris(hydroxymethyl)aminomethane–10 μ*M* cAMP) was incubated with 1 m*M* ethoxyformic anhydride for 5 min at 30° at the specified pH. Aliquots (25 μl) were assayed as described in the Experimental Section. The rate constants and pK_a were determined by the procedure of Burstein et al. (1974).

Table VII: Ethoxyformic Anhydride Inhibition of DEAE-Cellulose Bovine Muscle Protein Kinase I and II.[a]

	Enzyme Activity (pmol/min)	
	Kinase I	Kinase II
Control	130	200
Ethoxyformic anhydride (5 m*M*)	36	149
cAMP (10 μ*M*)	124	190
cAMP (10 μ*M*), ethoxyformic anhydride (5 m*M*)	3	21

[a] Bovine muscle protein kinase was prepared according to the methodology of Corbin et al. (1975). Modification by ethoxyformic anhydride was performed as described for Table I at the specified concentration of reagent. Enzyme solutions (1 mg/ml) were dialyzed for 14 hr prior to measuring enzyme activity. Aliquots (25 μl) were assayed for activity with cAMP (10 μ*M*) present as previously described (Witt and Roskoski, 1975b).

Discussion

Ethoxyformic anhydride inhibits the native bovine brain protein kinase holoenzyme in the presence, but not absence, of cAMP. It also inhibits the free catalytic subunit fraction. Inhibition by ethoxyformic anhydride shows pseudo-first-order kinetics, is associated with modification of a group with a pK_a of 7.8 ± 0.3, and is prevented by Mg^{2+}-ATP and the regulatory subunit. Protection by Mg^{2+}-ATP suggests that the modified residue associated with inactivation forms part of the active site. The finding that both Mg^{2+}-ATP substrate and regulatory subunit prevent inactivation suggests that these two substances interact with the same region of the active site. Protection by these two components is specific: other nucleotides exhibit modest or no protective effect and proteins (histone, phosvitin, casein) completely fail to protect.

Ethoxyformic anhydride reacts with several side chain residues such as phenolates, imidazoles, carboxylates, sulfhydryls, and amino groups (Larrouquère, 1964; Mühlrád and coworkers, 1969; Melchior and Fahrney, 1970). The pseudo-first-order kinetics of inactivation indicate that modification of a single residue is inhibitory. The failure of dilute hydroxylamine to regenerate activity provides evidence that the modified residue is not histidine (Burstein et al., 1974; Roskoski, 1974). The partial regeneration of activity by hydroxylamine suggests that modification of a tyrosine is associated with inhibition (Burstein et al., 1974). The apparent pK_a of 7.8 is low for tyrosine, but the reactive residue may be perturbed. Further experiments are required to definitely establish the identity of the residue whose modification is related to inhibition.

Blue Dextran affinity chromatography has been used to purify many enzymes including phosphofructokinase (Kopperschläger et al., 1971) and lactate dehydrogenase (Ryan and Vestling, 1974). Thompson et al. (1975) have proposed that the blue chromophore is a nucleotide analog which specifically complexes with those proteins containing the super-secondary structure known as the dinucleotide fold. Unlike holoenzyme, free catalytic subunit binds to Blue Dextran-Sepharose. The catalytic subunit can be eluted from the resin with low concentrations of ATP (0.5 m*M*). These results suggest that the ATP binding site of the catalytic subunit has the dinucleotide fold (Thompson et al., 1975). The cAMP binding site of the regulatory subunit is structurally distinct from the ATP site of the catalytic subunit since the former fails to bind to the affinity resin. Some catalytic activity does not bind to the affinity resin even

after chromatography. The inability of some activity to bind may be due to the presence of an enzyme with different structural characteristics. This suggests the possibility that protein kinases differ not only in regulatory subunit structure (Corbin et al., 1974), but also in catalytic subunit structure.

Unlike holoenzyme, the catalytic subunit is inhibited by ethoxyformic anhydride and also binds to Blue Dextran-Sepharose. Both Mg^{2+}-ATP and regulatory subunit prevent inhibition and binding. Taken together, these results support the hypothesis that the holoenzyme is inactive because the regulatory subunit shields the active site of the catalytic subunit. The failure of the regulatory subunit to recombine with and elute the catalytic subunit bound to Blue Dextran-Sepharose also supports the proposal that the regulatory subunit interacts at the active site. Similar results are observed with the bovine muscle type II protein kinase. On the other hand, the bovine muscle protein kinase I holoenzyme is inhibited by ethoxyformic anhydride and appears to bind to Blue Dextran-Sepharose in the absence of cAMP. Although the mechanism is unclear, Mg^{2+}-ATP stabilizes the protein kinase I holoenzyme. For example, in the presence of Mg^{2+}-ATP, holoenzyme from rabbit reticulocyte (I) resists heat denaturation (Tao, 1971) and that from rat heart (I) resists histone and NaCl dissociation (Corbin and coworkers, 1975). Mg^{2+}-ATP promotes reassociation of skeletal muscle (I) regulatory and catalytic units (Brostrom et al., 1971), prevents holoenzyme inactivation by ethoxyformic anhydride, and prevents the binding of holoenzyme to Blue Dextran-Sepharose. Further experiments are required to establish the nature of the difference between the interaction of the regulatory and catalytic subunits and the possible roles of Mg^{2+}-ATP in protein kinases I and II.

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