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Specific Binding of a Novel Compound, *N*-[2-(Methylamino)ethyl]-5-isoquinolinesulfonamide (H-8) to the Active Site of cAMP-Dependent Protein Kinase

MASATOSHI HAGIWARA, MASAKI INAGAKI, and HIROYOSHI HIDAKA

Department of Pharmacology, Mie University School of Medicine, Edobashi, Tsu, Mie 514, Japan

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SUMMARY

The interaction of the catalytic subunit of bovine cardiac muscle cAMP-dependent protein kinase with *N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide (H-8), the most potent and selective inhibitor toward cyclic nucleotide-dependent protein kinases in the series of isoquinolinesulfonamide derivatives, was studied. The addition of H-8 protected the catalytic subunit of the enzyme in a dose-dependent manner from irreversible inactivation by the ATP analogue *p*-fluorosulfonylbenzoyl-5′-adenosine (FSBA). The inactivation followed pseudo-first order kinetics and H-8 reduced the steady state constant of inactivation (K_i) without any effect on the first order rate constant (K_3). The quantitative binding of H-8 to the enzyme was measured under conditions of thermodynamic equilibrium using a gel filtration method. The catalytic subunit bound approximately 1 mol of drug/mol of protein with apparent half-maximal binding at 1.0 μM drug, whereas the enzyme irreversibly modified by FSBA did not bind the drug, confirming that the enzyme has no site for H-8 in the catalytic subunit other than the active site. The binding studies also showed that H-8 does not require divalent cations such as Mg^{2+} to bind to the catalytic subunit of the protein kinase. The binding of H-8 to the active site was characterized using FSBA and other affinity labeling reagents which have been postulated to modify residues at or near the active site of the catalytic subunit. H-8 protected the enzyme against inactivation by FSBA and Cibacron Blue F3GA but did not afford any protection against the covalent modification of 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) and 7-chloro-4-nitro-2,1,3-benzoxadiazole (NBD-Cl), suggesting that the binding site of H-8 does not involve the γ-subsite of the ATP binding site in the catalytic subunit, since DTNB and NBD-Cl are thought to modify the residues complementary to γ-phosphate of the ATP molecules.

Since the discovery of cAMP-dependent protein kinase (1) and the evidence that this protein kinase mediates the hormonal stimuli leading to a breakdown in glycogen, enzymatic phosphorylation, and dephosphorylation, attention has been directed to the possible molecular mechanisms of biological regulation by which intracellular events are controlled by external stimuli (2–4). The extracellular signals produce many of their biological responses by regulating the intracellular concentration of cyclic nucleotides, diacylglycerol, or Ca^{2+} (5, 6). The actions of these second messengers on biological pathways are achieved through the activation of specific cyclic nucleotide-dependent, Ca^{2+}/phospholipid-dependent, or Ca^{2+}/calmodulin-dependent protein phosphorylation.

In order to specify the function of each protein kinase, we synthesized a series of isoquinolinesulfonamide derivatives which directly inhibit protein kinase activities (7–9). Although these compounds inhibit phosphotransferase activities competitively with ATP, isoquinolinesulfonamides are structurally unrelated to nucleotides and specifically reduce certain kinase activity among many nucleotides utilizing enzymes.

The specificities of isoquinolinesulfonamides for protein kinases were thought to reflect the geometric difference of the active site among various protein kinases. To elucidate the mechanisms of isoquinolinesulfonamides to recognize the structural difference of protein kinases, protein-drug interactions were characterized using H-8, which is a specific inhibitor of cyclic nucleotide-dependent protein kinase, as a model case. cAMP-dependent protein kinase is composed of dissimilar regulatory and catalytic subunits, and the complete amino acid sequences of the bovine cardiac muscle type II regulatory subunit (10) and catalytic subunit (11) are known.

In the present study, we used the gel permeation technique employing high performance liquid chromatography to determine the binding constant of H-8 for the catalytic subunit, and

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ABBREVIATIONS: H-8, *N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); FSBA, *p*-fluorosulfonylbenzoyl-5′-adenosine; CBF3GA, Cibacron Blue F3GA; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

examined the requirement for a divalent metal cation (Mg^{2+}) for H-8 binding to the catalytic subunit. Another approach we used was chemical modification studies of the ATP-binding site. Since possible amino acid residues important to enzyme function have been assessed by chemical modification studies, we analyzed the competition of H-8 for these affinity labeling agents. Overall, these studies demonstrate interaction of the isoquinolinesulfonamide with the catalytic subunit of cAMP-dependent protein kinase and provide a basis for future studies of drug-binding sites on protein kinases.

Materials and Methods

Histone H2B, ATP, DTNB, and FSBA were obtained from Sigma Chemical Co., and CBF3GA was purchased from Ciba-Geigy. [γ-^{32}P]ATP was obtained from Amersham, England. The rest of the chemicals used in the work were reagent grade.

Enzyme Preparation and Assay

The catalytic subunit was prepared to apparent homogeneity as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using the procedure of Beavo *et al.* (12). cAMP-dependent protein kinase activity was assayed (9) in a reaction mixture containing, in a final volume of 0.2 ml, 25 mM Tris-HCl (pH 7.0), 10 mM magnesium acetate, 2 mM EGTA, 10 μM [γ-^{32}P]ATP (4×10^5 cpm), 0.5 μg of the enzyme, 400 μg of Histone H2B, and each compound, as indicated.

Chemical modification of the catalytic subunit

The bovine cardiac muscle catalytic subunit was chemically modified with either FSBA, CBF3GA, DTNB, NBD-Cl, or *o*-phthalaldehyde as follows.

FSBA. Catalytic subunit (0.05–0.5 mg/ml) was incubated with 0.2–5 mM FSBA at 25° in 50 mM potassium phosphate buffer, pH 6.8, containing 10% (v/v) dimethyl sulfoxide.

The reaction with FSBA was halted at the indicated times by removing aliquots of 20 μl from the reaction mixture and diluting them into 1 ml of an ice-cold buffer composed of 50 mM potassium phosphate, pH 6.8. A sample (20 μl) of this diluted enzyme was immediately assayed. The presence of diluted FSBA, after the reaction was stopped, caused less than 3% inactivation in the course of the manipulations involved.

CBF3GA. The catalytic subunit (0.05–0.5 mg/ml) was incubated with 0.1 mM CBF3GA in 50 mM potassium phosphate buffer (pH 6.8) at 25°. Portions were removed at various times for phosphotransferase actvity determinations.

DTNB. The catalytic subunit (0.05–0.5 mg/ml) was incubated with 0.2 mM DTNB at 25° in 50 mM potassium phosphate buffer (pH 6.8) containing 1% (v/v) ethanol. Portions were removed through the course of the reaction and assayed for catalytic subunit activity as previously described. Ethanol alone (1%) had no effect on enzyme activity.

NBD-Cl. The catalytic subunit (0.05–0.5 mg/ml) was incubated with 0.5 mM NBD-Cl in 50 mM potassium phosphate buffer (pH 6.8) at 25°. NBD-Cl was added as freshly prepared stock solution in acetonitrile. The final concentration of acetonitrile in the reaction mixtures was 1%; this concentration failed to alter catalytic subunit activity significantly.

***o*-Phthalaldehyde.** The catalytic subunit was incubated with 0.2 mM *o*-phthalaldehyde in 100 mM Hepes-NaOH, pH 7.3, and 1% methanol at 25°. *o*-Phthalaldehyde was prepared freshly in methanol prior to each experiment. Methanol alone (1%) had no effect on enzyme activity.

Equilibrium Binding of H-8

The equilibrium for the binding of H-8 by the catalytic subunit was measured by using gel exclusion chromatography as described by Hummel and Dreyer (13). Binding experiments were performed in buffer A (50 mM potassium phosphate, pH 6.8, 150 mM KCl) containing various concentrations of H-8. The catalytic subunit was dissolved in buffer A at a concentration of 10 μM (0.39 mg/ml) and the protein level was determined by the method of Lowry *et al.* (14). The concentration of H-8 was determined by the absorbance at 214 nm using $\epsilon^M = 40{,}300$. An aliquot (25 μl) of the enzyme solution was applied to a column (0.6 × 30 cm) of TSK-GEL G3000 SW which had been equilibrated previously in buffer A. The column was eluted with buffer A at a flow rate of 0.5 ml/min using a Jasco TRIROTAR III pump and absorbance of the effluent was monitored at 214 nm by using a Jasco UNIDEC-100-III detector. Absorbance signals were transmitted to a Jasco DP-L220 data processor.

Standard solutions of H-8 in buffer A for calibrations contained 0.3 and 30 μM H-8 above the concentration of H-8 in the buffer.

The trough area was measured by using two independent methods of calibration, external and internal, as described by Hummel and Dreyer (13). By addition of excess drug to the sample of protein, the trough was reduced or converted to a peak. The relationship between the area of these peaks or troughs and the amount of excess drug added to the sample was linear within the range of values used for this study. The amount of drug bound to protein is equal to the amount of excess drug required to bind the area of the trough to zero. The values for the amount of H-8 bound to the catalytic subunit calculated by external and internal calibrations differed by no more than 10% at each concentration of H-8.

Results

Inactivation of cAMP-dependent protein kinase catalytic subunit by FSBA. Purified catalytic subunit was inactivated upon incubation with FSBA at 25°. The time course of inactivation at 0.5 mM FSBA with various concentrations of H-8 is shown in Fig. 1. After 60 min incubation without H-8, greater than 95% of the original activity of the catalytic subunit was lost and with 10 μM H-8 about 75% of the original activity

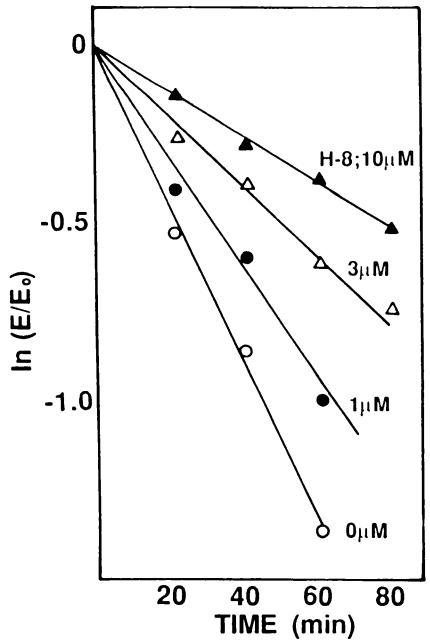

Fig. 1. Time course of inactivation of the catalytic subunit by FSBA in the presence of various concentrations of H-8. H-8 in concentrations ranging from 1 to 10 μM was included in the reaction mixture, and at indicated times an aliquot was removed and assayed as described under Materials and Methods. The natural logarithm of the residual enzyme activity was plotted against time. The H-8 concentration for each solution is indicated.

was maintained. A plot of natural logarithm of per cent residual activity versus time of incubation with FSBA gave a straight line. The slope of this line yields an apparent observed pseudo-first order rate constant (K_{obsd}) according to Eq. 1, where E is the activity at

$$-\ln (E/E_0) = K_{obsd}\, t \qquad (1)$$

any given time and E_0 is the activity at 0 time. This mechanism of inactivation is described by Eq. 2, where $E \cdot I$ is the concentration of the dissociable enzyme-FSBA complex, EI is the inactivated enzyme species, and K_3 is the first order rate constant of inactivation.

$$E + I \underset{K_2}{\overset{K_1}{\rightleftharpoons}} E \cdot I \xrightarrow{K_3} EI \qquad (2)$$

where $K_i = K_2/K_1$: the dissociation constant of FSBA for the enzyme. When K_{obsd}^{-1} was plotted against $[FSBA]^{-1}$ (Fig. 2), a straight line was obtained with a positive intercept on the *abscissa*. The maximum rate of inactivation (K_3) was 0.029 min^{-1}, and the K_i is 0.22 mM as determined from the *ordinate* and *abscissa* intercepts. The lines representing inactivation in the presence and absence of H-8 had a common ordinate intercept, thereby indicating that the maximum rate of inactivation (K_3) was unchanged. Analogous to competitive inhibition patterns obtained from double-reciprocal plots, this result indicates that the FSBA and H-8 compete for the same site.

Substrates and competitive inhibitors characteristically reduce the rate of enzyme inactivation by active site-directed reagents. The rate equaton is an extension of Eq. 2, which may be written as (15):

$$\frac{1}{K_{obsd}} = \frac{1}{K_3} + \frac{K_i}{K_3}1 + \frac{[S]}{K_a}\frac{1}{[I]} \qquad (3)$$

where S is a substrate or competitive inhibitor and K_a is its dissociation constant. From Eq. 3, the K_a of H-8 was 0.5–0.7 μM.

Equilibrium binding of H-8 to the catalytic subunit. The binding of H-8 to the catalytic subunit of cAMP-dependent protein kinase from bovine heart was measured using the gel permeation technique as described under Materials and Methods. Each protein sample with bound drug eluted as a sharp peak of absorbance in the excluded volume of the column with a retention time of 35 min (Fig. 3). The trough in Fig. 3A corresponded to the depletion of drug from the buffer due to drug binding by the protein, and the area of the trough represented the amount of bound drug. Fig. 3B shows the reduction of H-8 binding to the enzyme which was incubated for 60 min at 25° with 1 mM FSBA and filtrated on the G-50 column to eliminate free FSBA. The binding of H-8 to the C subunit is shown in Fig. 4 for a range (0.3–30 μM) of drug concentrations. Under our experimental conditions, bovine heart cAMP-dependent protein kinase catalytic subunit bound approximately 1 mol of drug/mol of protein with apparent half-maximal binding (K_m) at 1.0 μM H-8 (Fig. 4), whereas the enzyme affinity labeled by FSBA did not bind H-8. The K_m value of H-8 was unchanged in the presence or absence of magnesium ion (data not shown).

Effect of substrates and H-8 on inactivation by CBF3GA, FSBA, DTNB, NBD-Cl, and *o*-Phthalaldehyde. The rates of the inactivation of the catalytic subunit by 0.1 mM CBF3GA, 1 mM FSBA, 0.2 mM DTNB, 0.5 mM NBD-Cl, and 0.2 mM *o*-phthalaldehyde in the presence of various potential protecting reagents were examined. The inactivation reaction was carried out in the presence of 5 mM magnesium acetate. Table 1 summarizes the ability of ATP, protein substrates, and H-8 to protect the kinase molecule from reaction with these agents. The data show that 1 mM ATP protected the enzyme from all inactivating reactions but 1 mM H-8

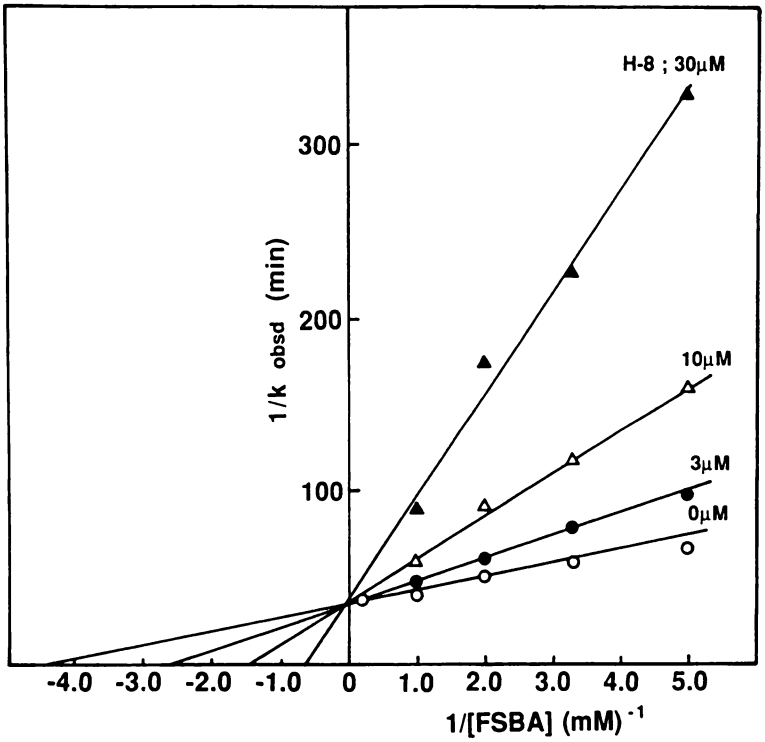

Fig. 2. Protection of the catalytic subunit by H-8 from inactivation of FSBA. The catalytic subunit (0.1 mg/ml) was incubated with varying concentrations of FSBA as described under Materials and Methods in the presence of various concentrations of H-8. Concentrations of H-8: none (○), 3 (●), 10 (△), and 30 (▲) μM. The value of K_{obsd} was determined at each FSBA concentration from the slope of the ln (E/E_0) versus time plot.

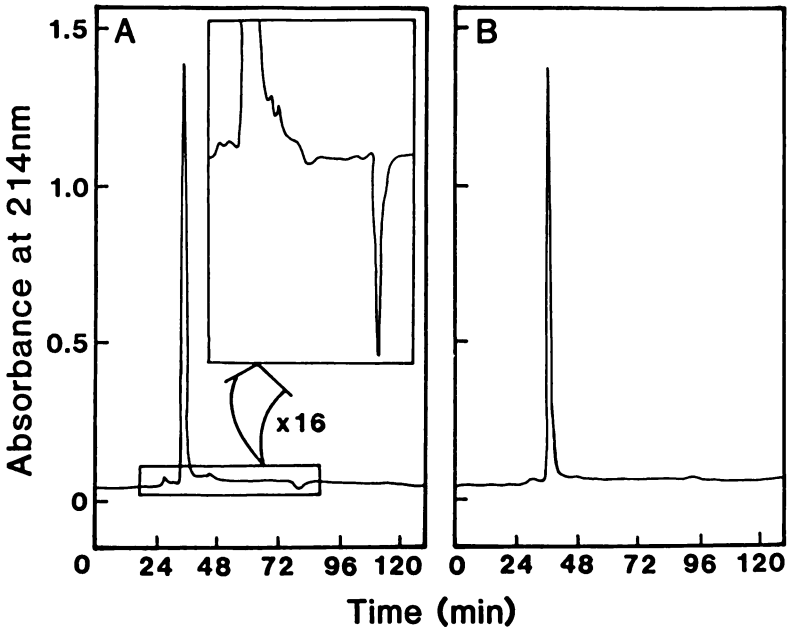

Fig. 3. Binding of H-8 to the catalytic subunit of cAMP-depenent protein kinase by high performance gel filtration liquid chromatography. Binding measurements were performed by the method of Hummel and Dreyer (13). The samples were applied to a column (0.6 × 30 cm) of TSK GEL G3000 SW equilibrated in 50 mM potassium phosphate, pH 6.8, 150 mM NaCl, and 1 mM at 0.5 ml/min. A. An aliquot (25 μl) of the catalytic subunit solution (10 μM) equilibrated in the column buffer was applied to the column and the elution was monitored with the absorbance at 214 nm. The *inset* is the same elution profile of which vertical range was 16 times longer. B. Twenty-five μl of the enzyme (10 μM), which was incubated for 60 min at 25° with 1 mM FSBA and filtrated on the G-50 column to eliminate free FSBA, were applied.

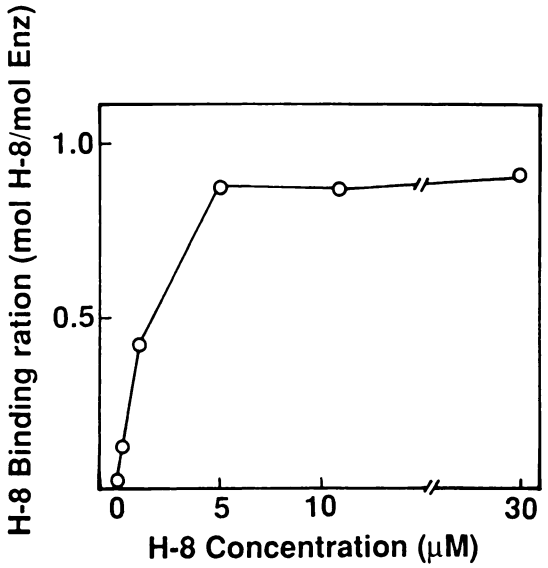

Fig. 4. Equilibrium binding of H-8 by the catalytic subunit of cAMP-dependent protein kinase as a function of H-8 concentration. The binding of H-8 to the enzyme was measured using the method of Hummel and Dreyer (13) in 50 mM potassium phosphate buffer (pH 6.8) containing 150 mM KCl. The *ordinate* expresses the number of mol of H-8 bound per mol of protein. The amount of enzyme molecule was calculated considering the lost activity through the penetration of the column. The *abscissa* shows the concentration of H-8. Results shown are the means of values calculated from external and internal calibrations, and the range of values was less than 10%.

provided no protection from the inactivation by DTNB, NBD-Cl, and *o*-phthalaldehyde. Histone H2B at concentrations of 2.0 mg/ml in the standard reaction mixture was ineffective in preventing inactivation by FSBA, DTNB, NBD-Cl, and *o*-phthalaldehyde, but was capable of protecting the enzyme from CBF3GA inactivation, as was whole casein at a concentration of 5 mg/ml.

Effect of nucleotides and Mg^{2+} on the inactivation of the catalytic subunit by FSBA and DTNB. Although H-8 could prevent FSBA-induced inactivation of the kinase molecule, it did not afford any protection from inactivation by DTNB. In an attempt to find out the subsite which H-8 occupies in the nucleotide site of the enzyme, we investigated the effect of ATP and some of its analogues, which are known to bind to this site, on the modification of the enzyme by FSBA and DTNB. The data showed that free nucleotide (without the presence of metal ion) failed to provide any protection (Table 2). Magnesium ion by itself did not protect the enzyme and, instead, accelerated the inactivation reaction by FSBA. H-8 (100 μM) was capable of fully protecting the enzyme from 1 mM FSBA inactivation in the absence of magnesium ion. In the presence of 10 mM magnesium acetate and an appropriate concentration of nucleotide, ATP, ADP, and AMP-PNP were capable of affording significant protection from inactivation by FSBA, but absence of the β-phosphate (AMP) resulted in an analogue that afforded no protection from inactivation whatsoever. A significant protection of DTNB reaction was observed only with two nucleotides containing the γ-phosphate group (ATP and AMP-PNP). In the absence of γ-phosphate (ADP), the protection was considerably lower.

Discussion

We found that some of the derivatives of *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) exhibited selective inhibition toward certain protein kinases (7, 8, 16), that H-8 was the most potent cyclic nucleotide-dependent protein kinase inhibitor in this series of compounds, and that the inhibition was freely reversible and of the competitive type with respect to ATP (9). To elucidate the mechanism of H-8 binding to the protein kinase, we examined the competition of H-8 with affinity labeling reagents at or near the ATP-binding site of the catalytic subunit. FSBA has been demonstrated to be an effective reagent for covalent labeling of the active center in the catalytic subunit of cAMP-dependent protein kinase II from bovine heart (17, 18). The structure of FSBA is similar to that of ATP except that the three phosphates have been replaced by a side chain of similar size, which contains a reactive sulfonyl fluoride group in the position of γ-phosphate. The

TABLE 1

Effect of ATP, H-8, and substrate proteins on inactivation of the catalytic subunit by various reagents

The catalytic subunit (0.1–0.2 mg/ml) was incubated with the specified concentration of reagent in 50 mM potassium phosphate buffer (pH 6.8) at 25° containing 10 mM magnesium acetate. Portions were removed for enzyme activity determinations as described under Materials and Methods. Residual activity at 35 min is given.

Addition to reaction mixture	0.1 mM CBF3GA	1 mM FSBA	0.2 mM DTNB	0.5 mM NBD-Cl	0.2 mM *o*-Phthalaldehyde[a]
Control	96	96	95	93	99
None	5	26	4	58	54
ATP (1 mM)	44	93	71	92	98
H-8 (1 mM)	98	98	5	65	55
Histone H2B (2 mg/ml)	97	20	4	60	56
Casein (5 mg/ml)	81	27	4	59	55

[a] Residual activity at 3 min is given.

TABLE 2

Effect of adenine nucleotides and H-8 in the presence or absence of Mg^{2+} on inactivation of the catalytic subunit by FSBA or DTNB

The catalytic subunit (0.1–0.2 mg/ml) was incubated with 1 mM FSBA or 0.2 mM DTNB at 25° in 50 mM potassium phosphate buffer (pH 6.8) as described under Materials and Methods. Mg^{2+} was added as the acetate salt. The phosphotransferase activity refers to that remaining after 30 min incubation.

Additions to assay mixture	% Residual activity			
	1 mM FSBA		0.2 mM DTNB	
	No Mg^{2+}	10 mM Mg^{2+}	No Mg^{2+}	10 mM Mg^{2+}
None	18	10	5	5
AMP (30 mM)	18	20	5	9
ADP (4 mM)	19	60	6	21
ATP (1 mM)	17	85	5	78
AMP-PNP (5 mM)	18	78	6	65
H-8 (0.1 mM)	100	98	14	13

kinetic analysis revealed that FSBA and H-8 compete for the same site of the kinase molecule and the dissociation constant of H-8 (K_a) was 0.5–0.7 μM, being consistent with the K_i value (1.2 μM) previously presented (9).

In order to exclude the possibility that the catalytic subunit has other binding sites for H-8 besides the ATP binding domain, the quantitative binding of this compound to the enzyme was measured under conditions of thermodynamic equilibrium by using a gel permeation technique of Hummel and Dreyer (13). We confirmed that the enzyme fully inactivated by FSBA no longer bound H-8. Under our experimental conditions, the enzyme lost 10–15% of original activity through the column. Considering the lost activity and the experimental error (<10%), 1 mol of the catalytic subunit bound 1 mol of drug with apparent half-maximal binding (K_m) at 1.1 μM. These results are in agreement with the K_a value as previously shown. The requirement for divalent metal cation (Mg^{2+}) for enzyme activity of the catalytic subunit is well established (17), and it has been demonstrated that metal ions can form complexes with the purine ring as well as with the phosphate groups of nucleotides (19). The result that the K_m of H-8 was not affected by the presence of magnesium indicated that H-8 did not require any metal ions to bind to the enzyme molecule. This feature of H-8 was confirmed from the result that H-8 was able to protect the enzyme from inactivation by FSBA in the absence of metal ion (Table 2). The metal ions, in the absence of nucleotide, may only weakly occupy the inhibitory site on the catalytic subunit and did not offer significant protection from chemical modification inactivation by FSBA or DTNB.

CBF3GA (20), FSBA (17, 18), DTNB (21, 22), NBD-Cl (23), and *o*-phthalaldehyde (24) were demonstrated to inactivate the enzyme by modifying the active site.

H-8 afforded full protection against modification by CBF3GA and FSBA, but not by DTNB, NBD-Cl, and *o*-phthalaldehyde. It has been previously shown that a cysteine (21–23) and a lysine (17, 18) residue at or near active site correspond to positions 199 and 72, respectively, in primary sequence of the catalytic subunit (10). FSBA covalently modifies the NH_2 group of Lys-72 concomitant with the loss of catalytic activity, whereas both NBD-Cl and DTNB alkylate SH groups of two cysteines (Cys-199 and Cys-343) of the catalytic subunit, and the loss of phosphotransferase activity was due to modification of only one cysteine residue at position 199 (25). *o*-Phthalaldehyde modifies both Lys-72 and Cys-199 simultaneously, and the proximal distance between the NH_2 function of Lys-72 and SH group of Cys-199 was estimated to be approximately 3 Å (24). However, Lys-72 and Cys-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 (23). It might be suggested that Lys-72 is the amino acid residue that binds to H-8 by considering the pattern of protection against FSBA or DTNB and NBD-Cl. However, H-8 does not affect the *o*-phthalaldehyde reaction which modifies Lys-72 and Cys-199 simultaneously.

The fluorescence-polarization binding studies suggest that modification of either Lys-72 or Cys-199 and -343 of the catalytic subunit does not affect the adenine- or ribose-binding regions of the active site of the enzyme but does alter the triphosphate-binding region on the catalytic subunit (26). Thus, it is interesting to note which ones among a number of ATP analogues are capable of affording protection from inactivation. In the presence of 10 mM Mg^{2+}, an appropriate excess of ATP analogue or H-8 was used (about 100 times its K_d value) (21) to rule out the possibility that the extent of the protection merely reflects the different affinities of the enzyme for each compound. In the series of ATP, AMP-PNP, ADP, and AMP, a significant protection of FSBA reaction was observed with the three nucleotides containing the β- and γ-phosphate groups (ATP, AMP-PNP, and ADP). But in the absence of γ-phosphate (ADP), the protection of DTNB was considerably lower as previously reported (22), and the absence of β-phosphate as well (AMP) resulted in an analogue that afforded no protection from inactivation by both FSBA and DTNB. The result that H-8 afforded full protection from FSBA inactivation but no protection of DTNB reaction suggests that H-8 is associated with the β-phosphate subsite as compared to the γ-phosphate subsite in the ATP-binding site of the kinase. The specificity of H-8 for cyclic nucleotide-dependent protein kinase might reflect the difference at or near the β-subsite of the ATP-

binding site between the cyclic nucleotide-dependent protein kinase and other kinases.

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References

1. Walsh, D. A., J. P. Perkins, and E. G. Krebs. An adenosine 3′,5′-monophosphate dependent protein kinase from rabbit skeletal muscle. *J. Biol. Chem.* **243:**3763–3765 (1968).
2. Rubin, C. S., and O. M. Rosen. Protein phosphorylation. *Annu. Rev. Biochem.* **44:**831–959 (1975).
3. Krueger, B. K., J. Forn, and P. Greengard. Depolarization-induced phosphorylation of specific proteins, mediated by calcium ion influx, in rat brain synaptosomes. *J. Biol. Chem.* **252:**2764–2773 (1977).
4. Greengard, P. Phosphorylated proteins as physiological effectors: protein phosphorylation may be a final common pathway for many biological regulatory agents. *Science* (*Wash. D.C.*) **199:**146–152 (1978).
5. Rasmussen, H., and D. B. P. Goodman. Relationships between calcium and cyclic nucleotide in cell activation. *Physiol. Rev.* **57:**421–509 (1977).
6. Nishizuka, Y. The role of protein kinase C in cell surface signal transduction and tumor promotion. *Nature* (*Lond.*) **308:**693–697 (1984).
7. Inagaki, M., S. Kawamoto, and H. Hidaka. Serotonin secretion from human platelets may be modified by Ca^{2+}-activated, phospholipid-dependent myosin phosphorylation. *J. Biol. Chem.* **259:**14321–14323 (1984).
8. Inagaki, M., M. Watanabe, and H. Hidaka. *N*-(2-Aminoethyl)-5-isoquinolinesulfonamide, a newly synthesized protein kinase inhibitor, functions as a ligand in affinity chromatography: purification of Ca^{2+}-activated, phospholipid-dependent and other protein kinase. *J. Biol. Chem.* **260:**2922–2925 (1985).
9. Hidaka, H., M. Inagaki, S. Kawamoto, and Y. Sasaki. Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. *Biochemistry* **23:**5036–5041 (1984).
10. Shoji, S., L. H. Ericsson, K. A. Walsh, E. H. Fischer, and K. Titani. Amino acid sequence of the catalytic subunit of bovine type II adenosine cyclic 3′,5′-phosphate dependent protein kinase. *Biochemistry* **22:**3702–3709 (1983).
11. Takio, K., S. B. Smith, E. G. Krebs, K. A. Walsh, and K. Titani. Primary structure of the regulatory subunit of type II cAMP-dependent protein kinase from bovine cardiac muscle. *Proc. Natl. Acad. Sci. USA* **79:**2544–2548 (1982).
12. Beavo, J. A., P. J. Bechtel, and E. G. Krebs. Preparation of homogeneous cyclic AMP-dependent protein kinase(s) and subunits from rabbit skeletal muscle. *Methods Enzymol.* **38:**299–308 (1978).
13. Hummel, J. P., and W. J. Dreyer. Measurement of protein-binding phenomena by gel filtration. *Biochim. Biophys. Acta* **63:**530–532 (1962).
14. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:**265–275 (1951).
15. Kitz, R., and I. B. Wilson. Esters of methanesulfonic acid as irreversible inhibitors of acetylcholinesterase. *J. Biol. Chem.* **237:**3245–3249 (1962).
16. Inagaki, M., S. Kawamoto, H. Itoh, M. Saitoh, M. Hagiwara, J. Takahashi, and H. Hidaka. Naphthalenesulfonamides as calmodulin antagonists and protein kinase inhibitors. *Mol. Pharmacol.* **29:**577–581 (1986).
17. Hixon, C. S., and E. G. Krebs. Affinity labeling of catalytic subunit of bovine heart muscle cyclic AMP-dependent protein kinase by 5′-*p*-fluorosulfonylbenzoyladenosine. *J. Biol. Chem.* **254:**7509–7514 (1979).
18. Zoller, M. J., and S. S. Taylor. Affinity labeling of the nucleotide binding site of the catalytic subunit of cAMP-dependent protein kinase using *p*-fluorosulfonyl-[^{14}C]benzoyl 5′-adenosine. *J. Biol. Chem.* **254:**8363–8368 (1979).
19. Cohn, M., and T. R. Hughes, Jr. Phosphorus magnetic resonance spectra of adenosine di- and triphosphate. *J. Biol. Chem.* **235:**3250–3253 (1960).
20. Witt, J. J., and R. Roskoski, Jr. Adenosine cyclic 3′,5′-monophosphate dependent protein kinase: active site directed inhibition by cibacron blue F3GA. *Biochemistry* **19:**143–148 (1980).
21. Armstrong, R. N., and E. T. Kaiser. Sulfhydryl group reactivity of adenosine 3′,5′-monophosphate dependent protein kinase from bovine heart: a probe of holoenzyme structure. *Biochemistry* **17:**2840–2845 (1978).
22. Jiménez, J. S., A. Kupfer, V. Gani, and S. Shaltiel. Salt-induced conformational changes in the catalytic subunit of adenosine cyclic 3′,5′-phosphate dependent protein kinase. Use for establishing a connection between one sulfhydryl group and the γ-P subsite in the ATP site of this subunit. *Biochemistry* **21:**1623–1630 (1982).
23. Hartl, F. T., and R. Roskoski, Jr. 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. *Biochemistry* **21:**5157–5183 (1982).
24. Puri, R. N., D. Bhatnagar, and R. Roskoski, Jr. Adenosine cyclic 3′,5′-monophosphate dependent protein kinase: fluorescent affinity labeling of the catalytic subunit from bovine skeletal muscle with *O*-phthalaldehyde. *Biochemistry* **24:**6499–6508 (1985).
25. Nelson, N., and S. S. Taylor. Selective protection of sulfohydryl groups in cAMP-dependent protein kinase II. *J. Biol. Chem.* **258:**10981–10987 (1983).
26. Bhatnagar, D., F. T. Hartl, R. Roskoski, Jr., A. L. Lessor, and N. J. Leonard. Adenosine cyclic 3′,5′-monophosphate dependent protein kinase: nucleotide binding to the chemically modified catalytic subunit. *Biochemistry* **22:**4350–4357 (1984).

Send reprint requests to: Dr. Hiroyoshi Hidaka, Department of Pharmacology, Mie University School of Medicine, Edobashi, Tsu, Mie 514, Japan.