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Affinity labeling of the catalytic subunit of cyclic AMP-dependent protein kinase by N^{α}-tosyl-L-lysine chloromethyl ketone

(active sites/hormone action/inhibitors/proteases/sulfhydryls)

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ABSTRACT **The catalytic subunit of cyclic AMP-dependent protein kinase (from rabbit skeletal muscle; ATP:protein phosphotransferase, EC 2.7.1.37) was found to be irreversibly inactivated by chloromethyl ketone derivatives of lysine and phenylalanine, chemical reagents originally designed for labeling the active sites of the proteolytic enzymes trypsin and chymotrypsin. This inactivation was shown to occur at pH 7.5 and 22°C, conditions under which chemically related alkylating reagents such as chloroacetamide and chloroacetic acid (which do not possess the amino acid side chain) fail to inactivate the enzyme. In the case of the chloromethyl ketone derivative of N^{α}-tosyl-L-lysine, the enzyme could be protected by its nucleotide substrate (MgATP), by one of its protein substrates (histone H2b), and by its regulatory subunit which, upon binding, shields the active site of the catalytic subunit. Differential labeling experiments, together with kinetic studies of the rates of modification of the sulfhydryl groups in the enzyme before and after inactivation with the chloromethyl ketone, suggest that the loss of activity is associated with one (kinetically characterized) sulfhydryl group present either at the active site of the enzyme or at a site intimately associated with it. The general implications of these results regarding the interpretation of affinity labeling experiments carried out in complex mixtures of proteins or under *in vivo* conditions are discussed.**

Since the discovery of cyclic AMP (cAMP)-dependent protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) (1) it has become evident that it plays a key role in mediating various hormonal effects and in controlling multiple metabolic processes (for reviews see refs. 2 and 3). This enzyme, found essentially in all mammalian tissues (4, 5), is composed of two types of subunits, one being catalytically active (C subunit) and the other (R) having a regulatory function. The inhibited form of the enzyme (R_2C_2) can be activated by cAMP according to the following equation (6–9):

$$R_2C_2 + 2cAMP \rightleftharpoons R_2(cAMP)_2 + 2C.$$

cAMP-dependent protein kinase is most likely capable of triggering different series of molecular events in different cells, resulting in the characteristic physiological response of each particular cell. It is therefore of interest to find out how the enzyme achieves specificity of action within the cell, which functional groups compose its catalytic and recognition sites, where the enzyme is located within the cell, and whether it becomes redistributed among the various subcellular organelles as a result of its stimulation. One approach to the elucidation of such questions makes use of active-site-directed irreversible inhibitors, which take advantage of biorecognition elements at the active site in order to label specifically some functional group(s) at the active site and preferably to restrict the chemical modification to a unique enzyme only. This paper describes the use of chloromethyl ketones of lysine and phenylalanine [TLCK and TPCK, originally designed (10) to selectively label the active sites of proteolytic enzymes] as active-site-directed labels for the C subunit of cAMP-dependent protein kinase. Our results assign a sulfhydryl group to the active site of the enzyme and kinetically characterize this sulfhydryl. The potential uses of these reagents are briefly discussed and the limitations of interpreting affinity labeling experiments carried out on complex mixtures of proteins or under *in vivo* conditions are pointed out.

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MATERIALS AND METHODS

Enzymes and Proteins. Pure C subunit of cAMP-dependent protein kinase was prepared from rabbit muscle by the method of Beavo *et al.* (11), the undissociated (inactive) form of the enzyme was obtained by the method of Hoppe and Wagner (12), and histone H2b was purified by the method of Böhm *et al.* (13).

Chemicals. [γ-^{32}P]ATP (2.0–2.7 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) was purchased from The Radiochemical Centre (Amersham, England). TLCK, TPCK, 5,5′-dithiobis(2-nitrobenzoic acid) (Nbs_2), Tris, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetracetic acid (EGTA), cAMP, ATP, and tosylfluoride were obtained from Sigma. Chloroacetamide and chloroacetic acid (both purchased from BDH) were recrystallized twice (from chloroform) before use. 2-(*N*-Morpholino)-ethanesulfonic acid (Mes) was obtained from Serva and $Mg(CH_3COO)_2$, from BDH.

Assay of cAMP-Dependent Protein Kinase. The assay was based on the phosphorylation of histone H2b with [γ-^{32}P]ATP (14). The reaction mixture (200 μl) contained the enzyme to be tested and the following ingredients at the indicated final concentrations: Mes (50 mM), $Mg(CH_3COO)_2$ (5 mM), EGTA (0.25 mM), histone H2b (1 mg/ml), and [γ-^{32}P]ATP (30–110 cpm/pmol; 245 μM) at pH 6.5. Assay mixtures used for the determination of enzyme activity in the presence of cAMP also contained this nucleotide in a final concentration of 5 μM. The assay was initiated by mixing an aliquot (50 μl, containing 50–100 ng of the C subunit) of the solution to be tested with the assay mixture (total volume, 200 μl). The reaction was allowed to proceed for 5 min at 30°C and was stopped by applying 100 μl of this reaction mixture onto a Whatman no. 3 filter paper (3 × 2.5 cm) which was immediately immersed in ice-cold 10%

Abbreviations: cAMP, adenosine 3′,5′-cyclic monophosphate; C subunit, catalytic subunit of cAMP-dependent protein kinase (type I); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); TLCK, N^{α}-tosyl-L-lysine chloromethyl ketone; TPCK, N^{α}-tosyl-L-phenylalanine chloromethyl ketone.

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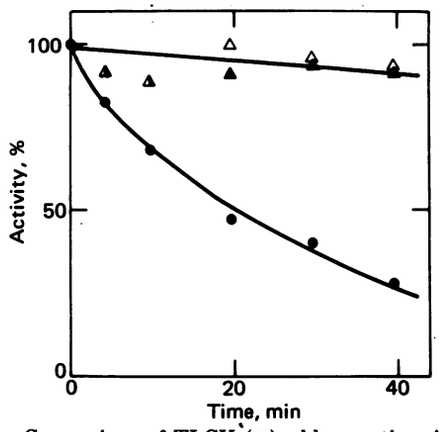

FIG. 1. Comparison of TLCK (●), chloroacetic acid (▲), and chloroacetamide (△) with regard to the inactivation of the C subunit. Each of the reaction mixtures (525 μl) contained 500 μl of pure C subunit (15 μg) dissolved in 50 mM Tris·HCl/0.5 mM EGTA, pH 7.5. Inactivation was initiated by addition of 25 μl of 20 mM inhibitor solution (to final concentration of 0.95 mM). The reaction was allowed to proceed at 22°C, and, at the indicated times, 50-μl aliquots were removed, diluted into 1 ml of 50 mM Mes/0.5 mM EGTA, pH 6.5, and then assayed immediately. The percentage of residual activity was calculated by comparison with a control incubation mixture in which the aliquot containing the alkylating reagent was replaced by 25 μl of water.

(wt/vol) trichloroacetic acid (the washing solvent was changed five times). After soaking in ethanol and ether, the filter papers were dried and assayed for radioactivity (in 10 ml of toluene-based scintillation fluid) with a Packard model 3003 Tri-Carb liquid scintillation spectrometer. The enzyme preparations used had a specific activity >3 units/mg (1 unit of enzyme activity is defined as the amount of enzyme that catalyzes the transfer, at pH 6.5 and 30°C, of 1 μmol of ^{32}P from [γ-^{32}P]ATP onto histone H2b per min).

Protein. Protein concentrations were determined by the method of Lowry *et al.* (15).

Chemical Modifications. Chemical modifications were carried out at enzyme concentrations >20 μg/ml to prevent inactivation of the enzyme during prolonged incubation at neutral or slightly alkaline pH (16). Under the conditions of our experiments, the loss of activity in control samples (without the modifying chemical reagent) was always <10%.

RESULTS AND DISCUSSION

Inactivation of the Catalytic Subunit of cAMP-Dependent Protein Kinase with TLCK, TPCK, and Analogous Alkylating Reagents. Results reported from several laboratories regarding the amino acid sequences around the site of phosphorylation in various protein and peptide substrates of cAMP-dependent protein kinase (17–24) indicate that basic and possibly hydrophobic amino acid residues provide important recognition elements in establishing the specificity of the enzyme. We therefore reasoned that TLCK and TPCK might be used as active-site-directed alkylating reagents for cAMP-dependent protein kinase. Indeed, the C subunit of this enzyme was rapidly inactivated by TLCK at pH 7.5, 22°C, conditions under which other alkylating reagents such as chloroacetamide and chloroacetic acid failed to inactivate the enzyme§ (Fig. 1). Chloroacetamide is certainly an adequate analog for TLCK as far as reactivity goes because it has a similar —CO—CH_2Cl group

Table 1. Inactivation of the C subunit by TLCK and TPCK and its prevention by MgATP and by histone H2b

Additions	% activity after exposure to alkylating reagent*	
	TPCK	TLCK
None	58	64
MgATP†	78	98
Histone H2b‡	45	90

* For 40 min at 22°C, under the experimental conditions described in the legend to Fig. 4. In each experiment, the concentration of TPCK was identical to that of TLCK.
† $Mg(CH_3COO)_2$, 9 mM; ATP, 0.22 mM.
‡ At 1 mg/ml.

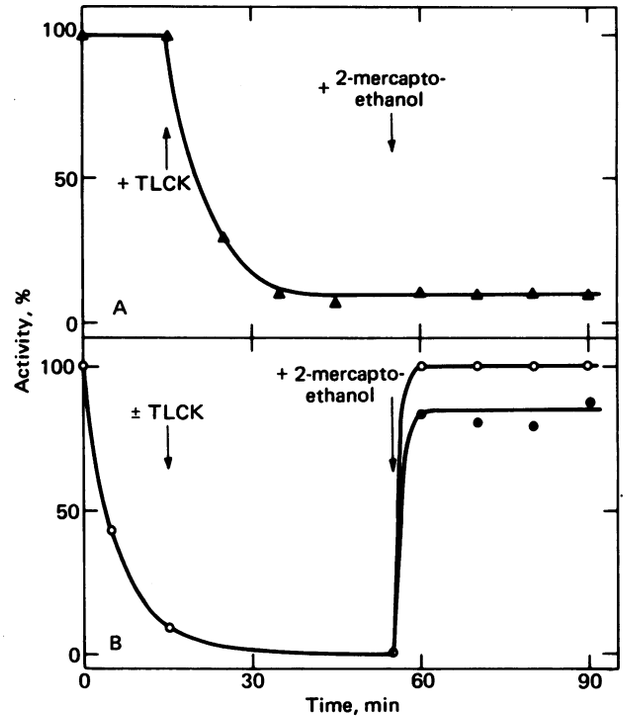

FIG. 2. Involvement of sulfhydryl(s) in the inactivation of the C subunit by TLCK as illustrated by differential labeling. (*A*) Kinetics of the inactivation of the enzyme with TLCK and lack of reactivation by 2-mercaptoethanol. The reaction mixtures (1.02 ml) contained 1 ml of pure catalytic subunit (34 μg) in 50 mM Tris·HCl/0.5 mM EGTA, pH 8.0 and were incubated for 15 min at 22°C; then inactivation was initiated by addition of an ethanolic solution of TLCK (20 μl; 50 mM) to bring the final concentration of the alkylating reagent to 1 mM. Reactivation was attempted by addition of 50 μl of 5M 2-mercaptoethanol. At the indicated times, 50-μl aliquots of the reaction mixtures were removed, diluted into 1 ml of 50 mM Mes/0.5 mM EGTA, pH 6.5, and assayed immediately. (*B*) Reaction of the enzyme with Nbs_2, exposure to TLCK, and quantitative reactivation by reaction with 2-mercaptoethanol. The reaction mixtures (1.1 ml) contained 1 ml of pure C subunit (39 μg) in the same buffer as in *A*. After addition of 100 μl of 0.25 mM Nbs_2 in the same buffer, the mixtures were incubated for 15 min at 22°C and then two 0.5-ml samples were removed from each reaction mixture and transferred to test tubes. To one sample (●) was added 10 μl of 50 mM ethanolic TLCK; to the other (○) was added 10 μl of ethanol (control). Both samples were then treated with 2-mercaptoethanol as in *A*. The reactions were allowed to proceed at 22°C, and at the indicated times 50-μl aliquots were removed, diluted as in *A*, and assayed immediately. The percentage of residual activity was calculated by comparison with an identical control incubation mixture that contained neither Nbs_2 nor TLCK.

§ These results are consistent with the finding by Sugden *et al.* (16) that iodoacetamide in the range of concentrations used in our experiments does not significantly inactivate the C subunit of the liver (type II) enzyme at pH 8.5.

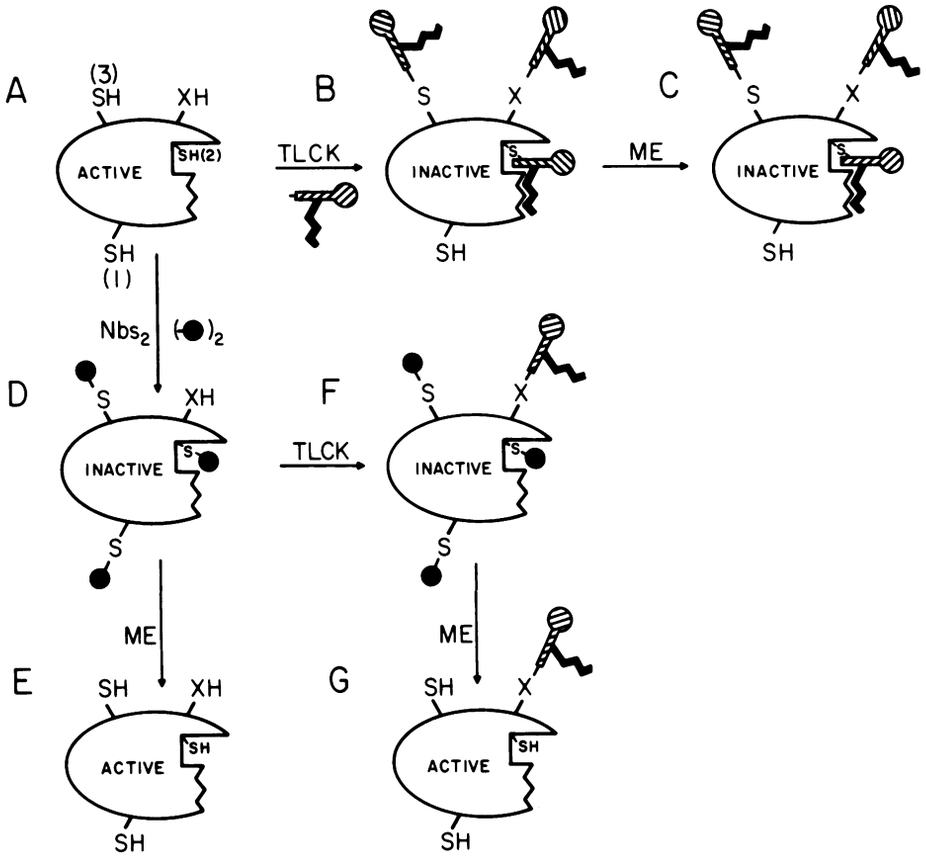

FIG. 3. Schematic representations. (*i*) Inactivation of the C subunit of cAMP-dependent protein kinase with Nbs_2 and the subsequent reactivation with 2-mercaptoethanol (ME) (pathway A → D → E). (*ii*) Inactivation of the C subunit with TLCK and the lack of reactivation upon exposure to 2-mercaptoethanol (pathway A → B → C). (*iii*) Shielding of sulfhydryl groups in the C subunit by Nbs_2, exposure to TLCK, and then reactivation of the enzyme by removal of the shielding Nbs groups with 2-mercaptoethanol (pathway A → D → F → G).

and it also is known to react with sulfhydryl compounds (e.g., cysteine) at a rate comparable with, if not higher than, the rate of reaction of TLCK with the same thiol compounds (25). Therefore, it seems that TLCK draws its enhanced reactivity toward the enzyme from a specific affinity for its target site, which is associated with the positively charged aliphatic side chain in TLCK.

TPCK also inactivates the C subunit of cAMP-dependent protein kinase at a rate similar to the rate of the TLCK inactivation (kinetics not illustrated). When the enzyme was treated with either one of the chloromethyl ketones (pH, 7.5–7.6, 40 min; 22°C), it lost 42% of its activity after reaction with TPCK and 36% after reaction with TLCK under similar conditions (Table 1).

The inactivation of the C subunit by TLCK and TPCK could not be attributed merely to a specific affinity of the enzyme for the tosyl moiety of these two reagents. This is indicated by the fact that, under the conditions of the experiment depicted in Fig. 1, tosylfluoride caused no inactivation of the enzyme, even when it was added in higher concentrations (up to 2 mM).

Differential Labeling Suggests the Involvement of Sulfhydryl Groups in the Inactivation. The C subunit of cAMP-dependent protein kinase [molecular weight, 40000 (26)] contains three half-cystines which can be determined as cysteic acid after oxidation with performic acid. It was previously shown that reaction of the C subunit with Nbs_2 inactivates the enzyme (27–30) and that this inactivation occurs concomitantly with the modification of 1 mol of SH groups per mol of the C subunit (27, 28). This inactivation of the enzyme was shown to be quantitatively reversible by reduction with 2-mercaptoethanol and to be due to a modification of one (or more) sulfhydryl group(s) present either at the active site or at another site intimately associated with it because the nucleotide substrate of the enzyme (MgATP) affords protection against inactivation, at concentrations (>10 μM) that correspond to the K_m value of the enzyme for MgATP (28).

In view of the fact that Nbs_2 is a group-specific reagent that reacts only with free sulfhydryl groups in proteins and because modification by it can be reversed by reaction with 2-mercaptoethanol, we attempted to establish by differential labeling whether the inactivation of the enzyme by TLCK is also associated with the same sulhydryl(s). When the C subunit was treated with Nbs_2 it was inactivated, and it then could be fully reactivated by addition of 2-mercaptoethanol (Fig. 2*B*) (A → D → E in Fig. 3). On the other hand, if the enzyme has reacted with TLCK, no reactivation can be achieved by reaction with 2-mercaptoethanol (Fig. 2*A*) (A → B → C in Fig. 3). If, however, the enzyme is first treated with Nbs_2 [to shield reversibly the sulfhydryl group(s) of the enzyme] and then treated with TLCK and subsequently exposed to 2-mercaptoethanol (A → D → F → G in Fig. 3), the enzyme regains ≈85% of its original activity (Fig. 2*B*). This experiment suggests that the functional group(s) responsible for the inactivation of the enzyme by TLCK can be protected reversibly by consecutive reaction with Nbs_2 and 2-mercaptoethanol—i.e., that they are sulfhydryl groups.

Attempts at Further Characterization of the Sulfhydryl Group(s) Responsible for the Inactivation. When C subunits of cAMP-dependent protein kinase were isolated in the presence of 2-mercaptoethanol, separated from the excess of low molecular weight thiol, and immediately titrated (in 100 mM of Hepes/1 mM EDTA, pH 7) at 22°C with a 50-fold excess of

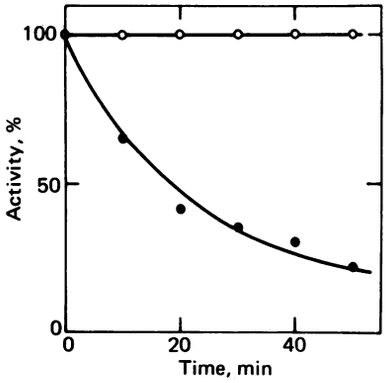

FIG. 4. Regulatory subunit protects the C subunit from inactivation by TLCK. The reaction mixtures (435 μl) contained 46 μg of the undissociated cAMP-dependent protein kinase dissolved in 50 mM Tris·HCl/0.5 mM EGTA, pH 8.0, with (●) or without (○) 11 μM cAMP. After preincubation for 15 min at 22°C, inactivation was attempted by addition of 10 μl of ethanolic 15 mM TLCK (final concentration, 0.34 mM). The reaction was allowed to proceed at 22°C and, at the indicated times. 50-μl aliquots were removed, diluted into 1 ml of 50 mM Mes/0.5 mM EGTA, pH 6.5, and assayed immediately. The percentage of residual activity was calculated in each case by comparison with a control incubation mixture in which the aliquot containing the alkylating reagent was replaced by 10 μl of ethanol. All the phosphotransferase activity assays were carried out in the presence of 5 μM cAMP.

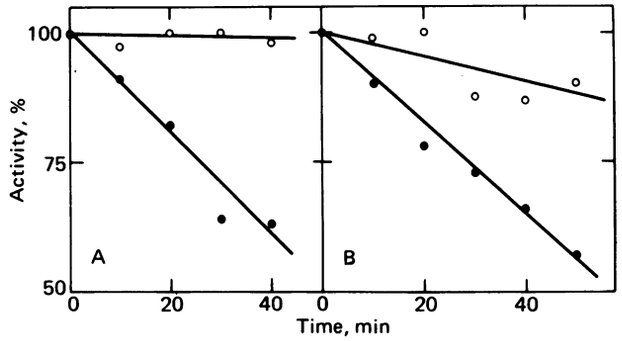

FIG. 5. Effect of the substrates MgATP and histone H2b on the inactivation of the C subunit by TLCK. (*A*) Protection by MgATP. Each of the reaction mixtures (560 μl) contained 16 μg of pure C subunit in 50 mM Tris·HCl/0.5 mM EGTA/9 mM $Mg(CH_3COO)_2$, pH 7.6, with (○) or without (●) 0.22 mM ATP. After preincubation for 15 min at 22°C, inactivation was attempted by addition of 10 μl of ethanolic 20 mM TLCK (final concentration, 0.35 mM). (*B*) Protection by histone H2b. Each of the reaction mixtures (560 μl) contained 9 μg of pure C subunit in 50 mM Tris·HCl/0.5 mM EGTA, pH 7.5, with (○) or without (●) histone H2b 1 mg/ml. After preincubation for 10 min at 22°C, inactivation was attempted by addition of 10 μl of ethanolic 17.5 mM TLCK (final concentration, 0.31 mM). In both *A* and *B* the reaction was allowed to proceed at 22°C and, at the indicated times, 50-μl aliquots were removed, diluted into 1 ml of 50 mM Mes/0.5 mM EGTA, pH 6.5, and then assayed immediately. The percentage of residual activity was calculated for *A* and *B* by comparison with control incubation mixtures in which the aliquot containing the alkylating reagent was replaced by 10 μl of ethanol.

Nbs_2, we found a mean (±SEM) of 2.3 ± 0.1 mol of sulfhydryl groups per mol of C subunits. Analysis of the kinetics of the reaction between Nbs_2 and the enzyme showed that the sulfhydryls numbered 1, 2 and 3 in Fig. 3 can be resolved into three classes which react with Nbs_2 at different rates. In a typical experiment carried out under the above conditions, 0.3 mol of sulfhydryl groups reacted with a rate constant $k_3 > 10{,}000\ M^{-1}\ min^{-1}$, 1.06 mol of sulfhydryl groups reacted with $k_2 = 3850\ M^{-1}\ min^{-1}$, and 0.94 mol of sulfhydryl groups reacted with $k_1 = 300\ M^{-1}\ min^{-1}$. It was also found that the inactivation of C was associated with the modification of 1 mol of sulfhydryl groups per mol of C subunits and that it occurred concomitantly with reaction of sulfhydryl number 2.

In the present study, with freshly prepared enzyme we found that upon reaction of a solution of C subunits (18 μM) with TLCK (4.5 mM) in 100 mM Hepes/1 mM EDTA, pH 7.0, after 30 min at 22°C 90% of the activity was lost. After removal of excess TLCK by gel filtration on Sephadex G-25 (in the same Hepes/EDTA buffer), the inactive enzyme still had 0.9 mol of free sulfhydryl groups per mol of C subunits. These residual sulfhydryl groups reacted with Nbs_2 with a rate constant of $530\ M^{-1}\ min^{-1}$, close to that of number 1 sulfhydryls (Fig. 3). The stoichiometry of inactivation, together with the correlation between inactivation and loss of number 2 sulfhydryls, suggests that TLCK inactivates the enzyme by modification of SH number 2 and that this SH group is either at the active site or at another site intimately associated with it. On the basis of the differential labeling experiment mentioned above, it can also be concluded that, even if TLCK reacts with some other functional group(s) in the protein (e.g., group X in Fig. 3), such a group is not likely to be associated with the inactivation of the enzyme.

Protection from Inactivation Afforded by the Regulatory Subunit and by the Nucleotide and Protein Substrates of the Enzyme. It was previously proposed that the regulatory subunit of cAMP-dependent protein kinase shields, at least in part, the active site on the catalytic subunits of the enzyme (31, 32). One would therefore expect that, if TLCK inactivates the enzyme by attaching itself to a sulfhydryl group at the active site of the enzyme, the regulatory subunit in the undissociated enzyme is likely to protect the C subunit from inactivation. Fig. 4 shows that this is indeed the case: TLCK failed to abolish the potential catalytic activity of the undissociated enzyme (measured after addition of cAMP) under conditions such that the dissociated enzyme is readily inactivated.

Furthermore, preincubation of the C subunit with either the nucleotide (MgATP) or a protein substrate (histone H2b) of the enzyme protected it from inactivation by TLCK (Fig. 5), thus fulfilling one of the important requirements of an active-site-directed affinity labeling reagent.

The regulatory subunit in undissociated cAMP-dependent protein kinase shielded the C subunit also from inactivation by TPCK (data not illustrated). However, in the case of TPCK the nucleotide substrate MgATP was much less effective in affording protection from inactivation, and, under the conditions of the experiment presented in Table 1, histone H2b did not afford any protection and even slightly accelerated the inactivation.

Implications of the Results. When dealing with a pure enzyme, the usefulness of active-site directed irreversible inhibitors is obvious because they constitute an important tool for mapping the enzyme active site—i.e., for identifying the functional groups involved in biorecognition and in the catalytic event. This mapping can be carried out by chemical studies in solution or by x-ray studies of stable enzyme–inhibitor complexes in the crystalline state.

When fluorescent or radioactively labeled, such inhibitors may also be useful for the localization of an enzyme within the cell, if they are capable of penetrating it. Likewise, these inhibitors may be used for the detection of physiologically important translocations, if any. In such cases it is imperative to ascertain, by additional independent techniques, that the labeling reagent is indeed specific in the given mixture of proteins and that its binding (under the experimental conditions used)

is restricted to the desired enzyme. The experiments presented here illustrate a case in which affinity-labeling reagents tailored to label the active site of two proteolytic enzymes (trypsin and chymotrypsin) were found to react with the active site of an enzyme that has an entirely different type of metabolic function.

Knowing the substrate specificity of cAMP-dependent protein kinase, it is easy to understand, by hindsight, why TLCK and TPCK could act as affinity labeling reagents for the C subunit of the enzyme, but this was not obvious *a priori*.

Now, following the impressive selectivity and discrimination achieved in the affinity labeling of functionally similar enzymes [e.g., the classical trypsin/chymotrypsin case (10)], several laboratories have used TLCK and TPCK to study complex biological processes such as fertilization, cell growth, protein synthesis, and virus maturation and also diseases such as emphysema and cancer (for a review see ref. 33). When such processes were affected, some authors suggested that a trypsin- or chymotrypsin-like protease is involved.

In view of the results reported here, such effects need not be ascribed to the inhibition of trypsin-like or chymotrypsin-like proteases but may well be due also to the inhibition of other, functionally unrelated enzymes (such as cAMP-dependent protein kinase) having at their active sites biorecognition elements similar to those of trypsin or chymotrypsin.

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