Sulfhydryl Group Reactivity of Adenosine 3′,5′-Monophosphate Dependent Protein Kinase from Bovine Heart: A Probe of Holoenzyme Structure†

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ABSTRACT: The spectrophotometric titration of SH groups in adenosine 3′,5′-monophosphate (cAMP) dependent protein kinase from bovine heart muscle with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) is described. The holoenzyme (R_2C_2) contains 16 SH groups, 12 of which react with DTNB in the native enzyme. The SH groups are distributed 2 per catalytic (C) and 4 per regulatory (R) subunit. The binding of cAMP to the holoenzyme or isolated R subunit prevents the reaction of one SH group per R subunit. Modification of SH groups, however, has only a small effect on cAMP binding to R. Reaction of the C subunit with DTNB results in >95% loss of catalytic activity. The kinetics of the DTNB reaction and the reversal of the inactivation process by treatment with dithiothreitol suggest that the inactivation is associated with SH group modification. Inactivation studies with the holoenzyme show that: (1) the R subunit inhibits DTNB inactivation of the C subunit in the absence of cAMP; (2) the rate of inactivation of the dephosphoholoenzyme in the presence of cAMP is considerably faster than that of the free catalytic subunit; and (3) the rate of inactivation of the phosphoholoenzyme in the presence of cAMP is faster than that of the C subunit but slower than the dephosphoholoenzyme. The results are interpreted as evidence for a significant interaction of the R and C subunits in the presence of saturating concentrations of cAMP. This interaction is modulated by the state of phosphorylation of R. To account for the inactivation data, a short-lived ternary complex containing R, C, and cAMP is postulated to be in rapid equilibrium with the subunits.

Adenosine 3′,5′-monophosphate dependent protein kinases (EC 2.7.1.37; ATP:protein phosphotransferase) are central to the mediation of the physiological effects of cAMP[1] in mammalian tissues (Krebs, 1972). The enzymes are classified into two main types (I and II) which differ primarily in the regulatory portion of the molecule (Hofmann et al., 1975). The protein kinase holoenzyme consists of regulatory (R) and catalytic (C) subunits in a quaternary complex (R_2C_2) of low catalytic efficiency. Activation of the phosphotransferase activity by cAMP is proposed to occur via dissociation of the C subunit from the holoenzyme after binding of cAMP to the R subunit (Brostrom et al., 1970). Furthermore, phosphorylation of the R subunit of the type II enzyme is reported to modulate its regulatory properties (Erlichman et al., 1974).

In addition to their obvious importance in the regulation of cellular metabolism, the cyclic nucleotide dependent protein kinases are of interest because they offer an excellent opportunity for investigation of both catalytic and control processes in enzyme action. We have undertaken studies which involve: (1) the kinetic (Moll & Kaiser, 1976) and chemical detection of intermediates in the catalytic and control mechanisms; and (2) the use of chemical probes to explore structure and function. In the present paper we report the results of an investigation into the effects of sulfhydryl group modification by 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) on a cAMP-dependent protein kinase from bovine heart muscle. We have quantitated and determined the reactivity of SH groups in the holoenzyme and isolated subunits. Our experiments on SH modification have allowed us to examine subunit interactions in the protein kinase holoenzyme.

Experimental Section

Materials

Histone IIA mixture and protamine sulfate (grade I) were obtained from Sigma. DEAE-Cellulose (Whatman DE 52) and cellulose phosphate paper P81 were acquired from Whatman, Inc. [γ-^{32}P]ATP and [8-^{3}H]cAMP were from Amersham/Searle. DTNB was obtained from Aldrich and recrystallized once from glacial acetic acid. Sephacryl S-200 and Sepharose 4B and 6B were obtained from Pharmacia. ω-Aminohexyl-Sepharose was prepared according to Shaltiel (1974). Millipore filters (HAWP) were from the Millipore Corp. All other biochemicals, reagents, and buffer salts were of the highest quality available.

Methods

Protein concentration was determined by the Lowry method (Lowry et al., 1951) with BSA as a standard. $NaDodSO_4$–polyacrylamide gel electrophoresis was performed according to Weber & Osborn (1969) on 10% gels. Protein samples were denatured in 1% $NaDodSO_4$, 1% 2-mercaptoethanol for 5 min at 100 °C. Gels were stained with Coomassie Blue.

Amino acid analyses were performed on a Beckman analyzer (Model 121) according to Moore & Stein (1963), after hydrolysis at 105 °C in 6 N HCl for 24, 48, and 72 h. Tryptophan was determined after 24-h hydrolysis at 105 °C with 4 N methanesulfonic acid containing 0.2% 3-(2-aminoethyl)-indole (Liu & Chang, 1971). Cysteine was analyzed as cysteic

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[1] Abbreviations used: cAMP, adenosine 3′,5′-monophosphate; cGMP, guanosine 3′,5′-monophosphate; $NaDodSO_4$, sodium dodecyl sulfate; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TNB, thionitrobenzoate anion of DTNB; R, regulatory subunit monomer; C, catalytic subunit of cAMP-dependent protein kinase; Mops, morpholinopropanesulfonic acid; DTT, dithiothreitol.

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acid (Hirs, 1967) or as carboxymethylcysteine (Crestfield et al., 1963).

Preparation of Protein Kinase. Bovine heart protein kinase was prepared by the method described by Rubin et al. (1974) with the following modifications. Sepharose 6B was substituted for Bio-Gel P-300 in the gel filtration step. The hydroxylapatite column was developed with a linear gradient of 50–250 mM KH_2PO_4 (pH 7.0). The holoenzyme obtained from the hydroxylapatite step was concentrated and dialyzed against 50 mM KH_2PO_4, 1 mM DTT (pH 7.0). The enzyme was then applied to a 2 × 13 cm bed of ω-aminohexyl-Sepharose equilibrated with the same buffer. The holoenzyme was eluted with a 400-mL linear gradient of 0–400 mM NaCl (Rangel-Aldao & Rosen, 1976a). The catalytic and regulatory subunits were prepared from the holoenzyme by fractionation on DEAE-cellulose in the presence of cAMP (Rubin et al., 1974). Removal of cAMP bound to R was facilitated by incubation with 4 mM cGMP at 25 °C for 30 min followed by dialysis for 48 h against four 1-L changes of 50 mM KH_2PO_4 (pH 7.5) at 4 °C. Phosphoholoenzyme was prepared as described by Hofmann et al. (1975).

Assays. Catalytic activity was assayed at 30 °C by the method of Witt & Roskoski (1975a). The incubation mixture contained 0.05–0.2 μg of holoenzyme or catalytic subunit, 25 mM Mops, 10 mM $MgSO_4$, 50 μM [γ-^{32}P]ATP, 125 μg of protaminesulfate or histone IIa, 2.5 mg/mL BSA with or without 1 μM cAMP, pH 7.0, in a final volume of 0.1 mL. Reactions were initiated by addition of [γ-^{32}P]ATP to the other reagents and terminated by spotting a 25-μL aliquot on cellulose phosphate paper. A unit of activity is defined as the number of nmol of ^{32}P transferred to protamine per min at 30 °C. Cyclic nucleotide binding was quantitated by the Millipore filter method (Gilman, 1970). The final incubation mixture contained 0.1–0.4 μM holoenzyme or R subunit, 25 mM Mops, 2.5 mg/mL BSA, and 50 μM [8-^{3}H]cAMP, pH 7.0, in a final volume of 50 μL. Binding was initiated by addition of enzyme. After incubation for 10 min at 25 °C, a 25-μL aliquot was spotted in the center of the Millipore filter which was rapidly washed with two 5-mL aliquots of cold buffer.

Sulfhydryl Group Titrations. The holoenzyme and subunits were prepared for titration of the SH groups by dialysis for 36 h against three 1-L changes of 50 mM KH_2PO_4—1 mM EDTA (pH 7.5) under bubbling N_2. No significant losses of the phophotransferase or cAMP binding activities were observed after extended dialysis of either the holoenzyme or subunits at concentrations of 1.5 mg/mL or greater. Titrations were monitored by observation of TNB anion formation at 412 nm on a Beckman M VI Acta spectrophotometer thermostated at 25 °C. In general, the holoenzyme or subunit (2–5 μM) in the appropriate buffer was placed in a 1-mL cuvette and titration was initiated by addition of excess DTNB in 50 μL of buffer. Sulfhydryl titers were calculated based on a molar extinction coefficient of 13600 M^{-1} cm^{-1} (Ellman, 1959) for the TNB anion. Titrations were performed in either 150 mM pyrophosphate–1 mM EDTA (pH 8.2) or 50 mM KH_2PO_4–1 mM EDTA (pH 7.5). Denatured proteins were titrated at pH 8.2 in the presence of 2% $NaDodSO_4$.

The kinetics of the reaction of DTNB with the catalytic subunit were performed at pH 7.5 with DTNB concentrations varying from 0.09 to 0.54 mM and the catalytic subunit at 1.8 or 3.6 μM. The kinetics of inactivation of the holoprotein kinase and the catalytic subunit were determined by assay of quenched aliquots of the reaction mixture. Quenching was accomplished by rapid addition of a 25-μL aliquot of the reaction mixture to 0.5 mL of 25 mM Mops (pH 7.0) containing 5 mg/mL BSA at 0 °C. Cyclic nucleotide concentration was maintained at 5 × 10^{-5} M or greater during the quenching process.

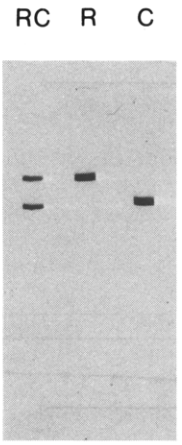

FIGURE 1: $NaDodSO_4$–polyacrylamide gel electrophoresis of 10 μg each, holoenzyme (RC), regulatory (R), and catalytic (C) subunits.

Rapid Gel-Filtration. Analytical gel filtration of native and modified protein kinase was done on a 1.6 × 91 cm bed of Sephacryl S-200 Superfine equilibrated with 50 mM KH_2PO_4, 1 mM EDTA, 50 mM KCl (pH 7.5). The activity profile of the modified enzyme was obtained after treatment of aliquots of the fractions with 10 mM DTT in the same buffer for 30 min at 25 °C. The extent of dissociation of the dephosphoholoenzyme due to cAMP binding was estimated by chromatography of 1 mL of a 3.2 μM solution of enzyme in the same buffer containing 0.5 mM cAMP. The chromatography was performed at 22 ± 1 °C and a flow rate of 10 mL cm^{-2} h^{-1}. At this flow rate a complete resolution of the holoenzyme (K_{av} = 0.08) and catalytic subunit (K_{av} = 0.36) was achieved in less than 6 h.

Results

The bovine heart cAMP-dependent protein kinase holoenzyme was purified to apparent homogeneity as judged by $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 1). The enzyme bound 2.0 ± 0.1 mol of cAMP/mol of holoenzyme and had a specific activity of 700 ± 70 units mg^{-1}. That the holoenzyme was largely in the dephospho form was demonstrated by the incorporation of 1.5 mol of ^{32}P per R subunit dimer from [γ-^{32}P]ATP under autophosphorylation conditions (Erlichman et al., 1974). The R and C subunits prepared from the holoenzyme were also homogeneous by $NaDodSO_4$ electrophoresis (Figure 1). The molecular weights of R and C calculated by calibration with standard proteins were 55 000 and 40 000, respectively, in excellent agreement with literature values (Hofmann et al., 1975). The R subunit bound 1.9 ± 0.1 mol of cAMP/R subunit dimer (mol wt 110 000). The isolated C subunit had a specific activity of 1100 ± 100 units mg^{-1}. Amino acid analysis of the catalytic subunit was in good agreement with that obtained by Demaille et al. (1977).

Titration of Denatured Protein Kinase with DTNB. The SH group titers determined for the protein kinase holoenzyme and the isolated subunits in 2% $NaDodSO_4$ are given in Table I. Under denaturing conditions 16 SH groups per holoenzyme react instantaneously with DTNB. The total number of SH groups determined by titration of the isolated subunits and of the holoenzyme are in excellent agreement. Two SH groups were found to be localized on each C subunit, while each R subunit contains 6 SH groups. Amino acid analysis of a hydrolysate of the catalytic subunit obtained after performic acid oxidation gave 3.5 mol of cysteic acid per mol. Reduction and

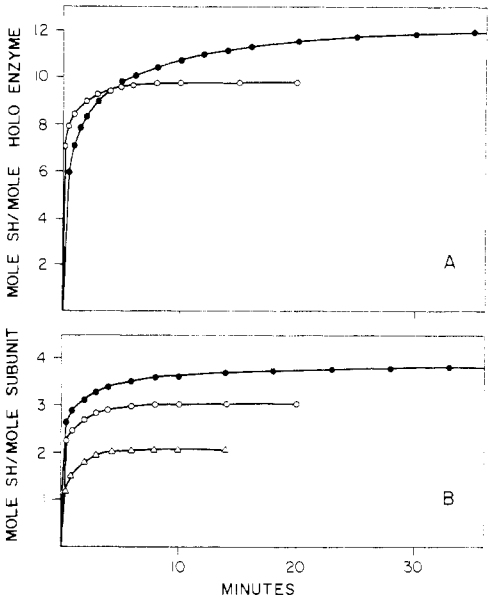

FIGURE 2: Typical time courses for the reaction of bovine heart protein kinase and isolated subunits with DTNB. (A) Protein kinase holoenzyme (0.41 mg/mL, 2.16 μM) was reacted with 0.36 mM DTNB at 25 °C (pH 8.2) in the absence (●) or presence (○) of 0.48 mM cAMP. Average SH group titers for three independent preparations were 12.1 ± 0.6 (− cAMP) and 9.9 ± 1.0 (+ cAMP) per mol of R_2C_2. (B) R subunit (0.17 mg/mL, 3.12 μM) was reacted as above in the absence (●) or presence (○) of cAMP. Average SH group titers were 4.0 ± 0.2 (− cAMP) and 3.2 ± 0.2 (+ cAMP) per mol of R. C subunit (△) (0.15 mg/mL, 3.86 μM) was reacted at pH 7.5 and 25 °C. Average SH group titer was 2.0 ± 0.1 per mol of C.

carboxymethylation, followed by acid hydrolysis, gave 1.8 mol of carboxymethylcysteine per mol of subunit.

The SH group titer of 2 mol/mol measured with DTNB for the catalytic subunit prepared as described is consistent with the amino acid analysis after carboxymethylation but is at variance with the performic acid oxidation results obtained in this work and by others (Demaille et al., 1977). In addition, Peters et al. (1977) have reported the titration of three SH groups on the bovine heart catalytic subunit using DTNB. Of the three groups one was found to be highly reactive toward DTNB at pH 7.0 and its modification did not greatly affect kinase activity. The discrepancy in SH group titers may be due to oxidation of this highly reactive group during the dialysis step of our isolation procedure. It should be noted, however, that no decreases (<10%) in SH group titers are observed when either the holoenzyme or subunits are stored for up to five days at pH 7.5 and 4 °C after removal of DTT by dialysis under N_2.

Titration of Native Protein Kinase and the Effects of cAMP. The quantitation of SH groups and the time course of DTNB titration of the native enzyme are shown in Figure 2. Comparison of Table I and Figure 2A show that the number of titratable SH groups on the holoenzyme changes from 16 in the presence of $NaDodSO_4$ to 12 in its absence. The SH group titers of the subunits in the absence of $NaDodSO_4$ (Figure 2B) indicate the groups exposed by $NaDodSO_4$ are localized on the R subunit.

From the results illustrated in Figure 2A it is apparent that a saturating (0.5 mM) concentration of cAMP both accelerates the rate of SH group titration and decreases the titer. The SH group titer is reduced by 2.2 ± 0.4 mol of SH/mol of holoenzyme or about one SH per cAMP binding site. The time dependence of reaction of DTNB with the isolated subunits is shown in Figure 2B. In the absence of cAMP 4 SH groups

TABLE I: Total Sulfhydryl Groups of Protein Kinase Determined by DTNB Titration in the Presence of Sodium Dodecyl Sulfate.[a]

	Mol of SH/mol[b] of protein	Integer value
Holoenzyme	14.9 ± 1.0	14-16
Regulatory subunit	5.8 ± 0.2	6
Catalytic subunit	2.1 ± 0.1	2
Total for R_2C_2[c]	15.8 ± 0.6	16

[a] Experimental conditions given under Methods. [b] Average of two independent determinations on two different preparations. [c] Determined by summation of subunit titers.

react per R subunit monomer. The presence of cAMP prevents the titration of 0.8 ± 0.2 SH groups per R subunit.

Although cAMP prevents the titration of one SH group per bound cAMP, modification by DTNB has only a modest effect on cAMP binding. When either the holoenzyme or the R subunit is completely reacted with DTNB in the absence of cAMP, the modified species remain competent in binding cAMP. Under conditions where the cyclic nucleotide saturates the unmodified enzyme, the fully modified holoenzyme (12 SH groups reacted) binds 1.5 ± 0.2 mol of cAMP/mol. The same result is obtained with the isolated R subunit which binds 1.0 ± 0.1 mol of cAMP/mol of R subunit dimer after complete modification.

In the absence of cAMP, reaction of all 12 available SH groups in the holoenzyme with DTNB may occur with concomitant dissociation to the subunits, since the total SH titer of the holoenzyme agrees well with the combined SH group titers of the isolated subunits. The observation that 80% of the catalytic activity appears at the elution volume of the catalytic subunit when the holoenzyme is completely modified with DTNB at pH 8.2 and chromatographed on Sephacryl S-200 supports this suggestion. The remaining 20% of activity is eluted as intact holoenzyme.

Kinetics of the Reaction of DTNB with the Catalytic Subunit. At pH 8.2 the reaction of the two sulfhydryl groups on the catalytic subunit with the concentrations of DTNB employed is instantaneous on the time scale of a conventional recording spectrophotometer. At pH 7.5, however, the reaction is slow enough to be monitored by conventional spectrophotometric techniques (see Figure 2B). The formation of TNB at pH 7.5 using DTNB at a concentration in 20-fold excess over total concentration of SH groups follows biphasic kinetics as illustrated in Figure 3A. Extrapolation of the reaction trace in the slow phase to the ordinate intercept of the semilog plot and a replot of the fast reaction data shows that 1 equiv of TNB is formed in each phase. This result is consistent with the titration of two distinct SH groups per subunit.

Nonlinear regression fitting of the time course data to eq 1 gave observed values of 1.2 ± 0.1 mol of S_sH and 0.8 ± 0.1 mol of S_fH per mol of catalytic subunit. The linear dependence of the observed rate constants (eq 1) on the DTNB concentration was used to calculate the second-order rate constants (k_f and k_s) for the fast and slow reacting SH groups. This analysis gave $k_s = (2.67 \pm 0.17) \times 10^3\ M^{-1}\ min^{-1}$. The rate of the fast reaction was such that only the last 20–40% was observed, and k_f could only be estimated to be approximately 10 k_s.

$$[TNB]_t = [TNB]_\infty - [S_fH]e^{-k_f^{\circ}t} - [S_sH]e^{-k_s^{\circ}t} \quad (1)$$

The addition of MgATP to the catalytic subunit had a significant effect on the kinetics of SH titration. When the catalytic subunit was titrated with DTNB in the presence of 0.5

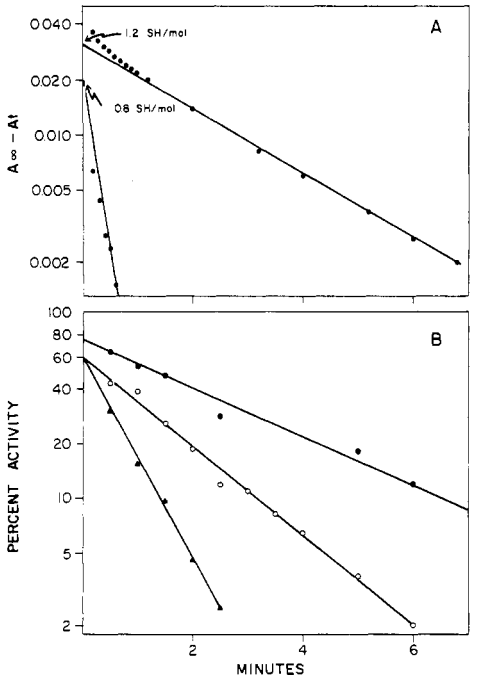

FIGURE 3: (A) Typical first-order plot of the reaction of DTNB with the catalytic subunit. Extrapolation of the measurements in the slow phase to zero time gives 1.2 SH per mol of C. A replot (lower trace) of the data of the fast phase corresponds to 0.8 SH per mol of C. (B) First-order plots for the inactivation of the catalytic subunit by DTNB. Catalytic subunit (1.93 μM) in 50 mM KH_2PO_4–1 mM EDTA (pH 7.5) was incubated at 25 °C with 0.45 mM (▲), 0.19 mM (○), and 0.09 mM (●) DTNB. At periodic intervals a 25-μL aliquot was withdrawn and quenched as described in the Experimental Section. Twenty-five microliters of the quenched solution was assayed within 30 min. Lines are least-squares fits to the experimental points.

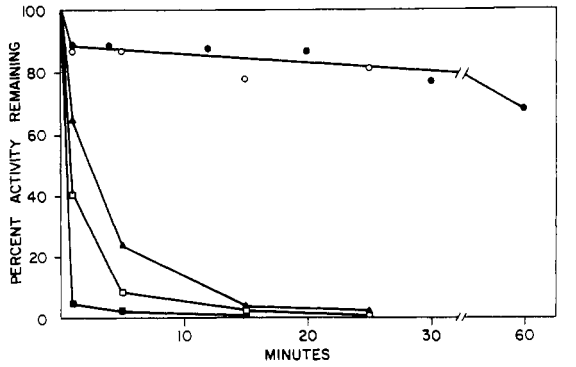

FIGURE 4: Time course for the inactivation of protein kinase holoenzyme by DTNB under pseudo-first-order conditions. Dephosphoholoenzyme (●), 2.41 μM, and phosphoholoenzyme (○), 1.20 μM, were incubated with 0.36 mM DTNB at pH 7.5 in the absence of cAMP, quenched, and assayed in the presence of cAMP. Dephosphoholoenzyme (■), 1.20 μM, phosphoholoenzyme (□), 1.20 μM, and C subunit (▲), 1.95 μM, were preincubated with 0.5 mM cAMP for 15 min at 25 °C and reacted with 0.12 mM DTNB at pH 7.5, quenched, and assayed.

mM ATP and 10 mM Mg^{2+}, no change in SH titer was observed, and the time course of the reaction became monophasic. The second-order rate constant measured for the reaction was $(5.95 \pm 0.32) \times 10^2$ M^{-1} min^{-1}. Thus the addition of MgATP at relatively high concentration seems to lower the reactivity of both SH groups on the C subunit toward DTNB so that $k_f \cong k_s$.

Inactivation of the Catalytic Subunit and the Holoenzyme by DTNB. Incubation of the catalytic subunit with excess DTNB at pH 7.5 resulted in a 95–98% loss of phosphotransferase activity employing either protamine sulfate or histone IIA as the substrate. Incubation of the inactivated subunit with 10 mM DTT at pH 7.5 restored 90–95% of the original activity. The kinetics of inactivation of the C subunit followed pseudo-first-order kinetics as shown in Figure 3B. Extrapolation of the first-order plots to zero time indicates that the inactivation is biphasic with between 25 and 40% of the catalytic activity lost in the first 30 s. The slow reaction which accounts for 60–75% of the activity lost is characterized by a second-order rate constant of $(2.89 \pm 0.44) \times 10^3$ M^{-1} min^{-1}. This value is within experimental error of the k_s value of $(2.67 \pm 0.17) \times 10^3$ M^{-1} min^{-1} determined from the kinetics of TNB formation.

The time dependence of the inactivation of the holoenzyme by DTNB at pH 7.5 in the absence and presence of cAMP is compared with that of the inactivation of the C subunit in Figure 4. In the absence of cAMP the regulatory subunit inhibits the inactivation of the catalytic subunit. The phospho- and dephosphoholoenzyme retain >70% of their original activity under reaction conditions which result in complete inactivation of C. When TNB formation was monitored at pH 7.5, 25 °C, and 0.36 mM DTNB the time required for complete titration (12 SH groups) of the holoenzyme was >12 h. Thus in the holoenzyme complex the reaction of the SH groups responsible for loss of phosphotransferase activity is greatly inhibited.

The inactivation of the dephosphoholoenzyme under the same pseudo-first-order conditions, but in the presence of cAMP, appears to be instantaneous within the time limits of our measurements as shown in Figure 4. The dephosphoholoenzyme reconstituted from the isolated subunits shows the same behavior. Preincubation of the holoenzyme for up to 2 h with cAMP has no effect on the subsequent inactivation. The rate of inactivation of the catalytic subunit alone is unaffected by addition of cAMP. When the DTNB concentration was lowered to 12 μM, the time course of the inactivation of the holoenzyme (0.3 μM) in the presence of cAMP was still too rapid to follow with >75% loss of activity in the first minute. The DTNB inactivation of the dephosphoholoenzyme in the presence of cAMP is, therefore, an extremely facile process.

The inactivation of the phosphoholoenzyme in the presence of cAMP proceeds more rapidly than that of the free catalytic subunit but at a more moderate rate than seen with the dephosphoholoenzyme (Figure 4). If no interaction between R and C exists in the presence of saturating concentrations of cAMP, the rate of holoenzyme inactivations should be the same as that of the free catalytic subunit. This is not the case since the presence of regulatory subunit and its phosphorylation state greatly affects the inactivation kinetics.

Previous investigators (Erlichman et al., 1974) found that the dephosphoholoenzyme was not dissociated by cAMP addition under conditions where the phosphoenzyme was dissociated as judged by gel-filtration chromatography. This result suggested that the accelerated inactivation reaction seen in the presence of cAMP might occur through an undissociated holoenzyme·cAMP complex. Rapid gel filtration chromatography under conditions that closely mimic those used for the inactivation process was performed to detect undissociated enzyme. Chromatography of the dephosphoholoenzyme in the presence of 0.5 mM cAMP at pH 7.5 results in elution of >99.5% of the catalytic activity at the elution volume of the catalytic subunit, indicating virtually complete dissociation of the holoenzyme into its subunits. The inactivation process thus must occur through some transient species involving the regulatory subunit.

Discussion

Spectrophotometric titration of sulfhydryl groups in Na-DodSO$_4$-denatured bovine heart muscle cAMP-dependent protein kinase with DTNB reveals a total of 16 SH groups present per holoenzyme tetramer (R_2C_2, mol wt 190 000). Twelve of the SH moieties are available for titration with DTNB in the native holoenzyme. The available SH groups are distributed four per R subunit monomer (mol wt 55 000) and two per C subunit (mol wt 40 000). Although the reaction of all 12 SH groups on the holoenzyme occurs only with substantial disruption of the quaternary structure, their reactivity with DTNB has allowed us to probe subunit and substrate interactions relevant to the catalytic and regulatory mechanisms of the enzyme.

The evidence presented here suggests that sulfhydryl groups on the holoenzyme or R subunit are not directly involved in cAMP binding to the enzyme. Although saturating concentrations of cAMP prevent the disulfide exchange reaction of one SH group per R subunit, the modification, in the absence of cAMP, of all available SH groups on either the dephosphoholoenzyme or R subunit has only a nominal effect on the subsequent ability to bind cAMP. The change in SH titer with cAMP present may be the result of a conformational change in the R subunit. Alternatively, it might be argued that bound cAMP simply sterically blocks the approach of DTNB to one SH group on each R subunit. Since the size of the TNB moiety covalently attached (in the absence of cAMP) to the SH group in question is approximately half that of DTNB the observation that the fully modified R subunit can bind cAMP does not preclude the possibility of direct steric hindrance of the approach of DTNB by the cyclic nucleotide. However, taken together with the dramatic effects of cAMP on holoenzyme structure (Erlichman et al., 1971; Witt & Roskoski, 1975b) the decrease in SH titer seems most reasonably attributed to a conformational change in the R subunit. Thus, our results provide chemical evidence for a cAMP induced conformational change in the regulatory subunit of a cAMP-dependent protein kinase.

The catalytic subunit of the bovine heart enzyme as prepared here appears to contain two sulfhydryl groups, as indicated by both the amount of TNB formed and the kinetics of its formation when C is reacted with DTNB. The biphasic inactivation seen indicates that modification of either SH group with DTNB effects phosphotransferase catalysis. That the loss of catalytic activity is due to SH modification is supported by: (1) the identity of the second-order rate constants for TNB formation and C subunit inactivation in the slow phase and (2) the complete reversal of inactivation by dithiothreitol. During the course of this work, a report on the biphasic inactivation of the bovine heart catalytic subunit by DTNB at pH 7.0 appeared (Peters et al., 1977). Our observations on the inactivation are essentially identical with those reported with the exception that we did not encounter a problem with subunit precipitation at pH 7.5.

As pointed out by Peters et al. (1977) the observation that modification of two SH groups is required for diminution of nearly all of the catalytic activity indicates that neither one of these groups by itself is essential to the catalytic process. Previous reports concerning SH group modification of catalytic subunits derived from type I (Bechtel et al., 1977) and type II (Sugden et al., 1976) holoenzymes support this conclusion. The mechanism by which the DTNB modification inhibits catalysts of phosphate transfer is not clear. To at least some extent it must be due to the size of the TNB moieties covalently bound since smaller groups are less effective inhibitors (Peters et al., 1977). Inactivation of the type II subunit from bovine liver by iodoacetamide is slowed by addition of MgATP. This has been proposed to occur by either a MgATP induced conformational change in enzyme structure or direct steric interference with the alkylation reaction (Sugden et al., 1976). In the present case MgATP decreases the reactivity of the SH groups toward DTNB. Ultimately, however, both SH groups react, albeit at a considerably reduced rate. This might be considered evidence that both SH moieties are at or near the MgATP binding site. The situation is, on the other hand, somewhat reminiscent of the results obtained in SH modification studies on rabbit phosphofructokinase in which it was concluded that loss of enzymatic activity did not occur by direct active site modification or a major conformational change in the enzyme (Schwartz et al., 1976).

The very slow inactivation of holoenzyme in the absence of cAMP clearly demonstrates that the regulatory subunit inhibits the reaction of at least one SH group on the catalytic subunit at pH 7.5. It has been postulated that R subunit inhibition of type II holoenzyme from bovine brain occurs through shielding of the MgATP binding site on the C subunit (Witt & Roskowski, 1975b). However, the observed intramolecular autophosphorylation (Rangel-Aldao & Rosen, 1976b) of the bovine heart holoenzyme, which occurs in the absence of cAMP, suggests that the MgATP binding site is available or "open" in the holoenzyme complex. We see no compelling reason at this point to assume the presence of a distinct MgATP binding site for the phosphorylation of exogeneous substrates by the catalytic subunit. It has been suggested (Demaille et al., 1977) that inhibition of the C subunit by the R subunit and a protein kinase inhibitor protein is, in part, similar in that they both shield the protein substrate binding site on the C subunit. In the light of this suggestion the efficacy of the R subunit in protecting the C subunit from reaction with DTNB may mean that the protein binding site is primarily involved in the inactivation process. The bulky TNB moieties might protrude into the protein substrate binding site.

The remarkably facile inactivation of the dephosphoholoenzyme by DTNB in the presence of saturating concentrations of cAMP can only reasonably be explained by an intimate interaction between the R and C subunits under the conditions employed. The dependence of the inactivation kinetics on the phosphorylation state of R also requires involvement of the R subunit in the accelerated loss of activity. The inactivation data can be rationalized by invoking the presence of a ternary complex composed of R, C, and cAMP (I of eq 2). The possibility of the presence of a substantial concentration of I which is altered by the phosphorylation state of R seems unlikely in view of our gel filtration results. The previous observation of undissociated dephosphoenzyme in the presence of cAMP (Erlichman et al., 1974) was probably due to the low concentration of cAMP employed.

$$R_2C_2 + cAMP \rightleftarrows \underset{I}{R_2(cAMP)_2C_2} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} R_2(cAMP)_2 + 2C \qquad (2)$$

The phosphorylation of R has been reported to slow the reassociation of R with C (Rangel-Aldao & Rosen, 1976a) and to increase its affinity for cAMP (Hofmann et al., 1975). In the former report it is suggested that phosphorylation of R does not change the dissociation constant for cAMP. The reason for the discrepancy in these reports with regard to cAMP binding is unclear and may be related to the different enzyme concentration, temperature, and methods of analysis employed. A more recent report on subunit reassociation (Rangel-Aldao

& Rosen, 1977) suggested that 50% reassociation of the dephosphoenzyme occurs at a concentration of cAMP tenfold higher than that showing a similar effect on the phosphoenzyme. This analysis was based on kinetic experiments and it is unclear whether equilibrium conditions were obtained. If, however, it is assumed that the affinity of phospho- and dephospho-R for cAMP is the same and that equilibrium was achieved, then the observations on reassociation strongly suggest the existence of a ternary complex such as I. Moreover, the behavior of the cAMP analogue cyclic adenosine 3',5'-phosphorothiolate in the activation of protein kinase suggests that cAMP binding and subunit dissociation are not necessarily concerted (Eckstein et al., 1974).

Our inactivation results may then be explained by the postulation of transient ternary complexes such as I in which the SH group(s) on the C subunit is activated toward reaction with DTNB. The slower rate of inactivation of the phosphoenzyme in the presence of cAMP when compared with the dephosphoenzyme may be the result of a smaller rate constant (k_{-1}) for recombination of phospho-R(cAMP) with C. It also remains possible that the rapid inactivation of C in the ternary complex, I, is not caused by direct reaction of C with DTNB. The reaction may occur in a stepwise process with the reaction of DTNB with free $R_2(cAMP)_2$ first, followed by subunit recombination and intracomplex transfer of TNB from R to C.

Although the exact mechanism by which inactivation of the holoenzyme occurs is unclear, it is obvious that the catalytic and regulatory subunits of cAMP-dependent protein kinase communicate in solution in the presence of high concentrations of cAMP. Our chemical modification results are consistent with previous contentions that the phosphorylation of R modulates its interaction with C in type II protein kinases. We are continuing to study the mechanism of the inactivation of the catalytic subunit and the structural factors involved. Finally, a knowledge of the reactivity of the SH groups in the protein kinase should permit the attachment of reporter groups to specific sites allowing us to probe further the subunit and cAMP–protein interactions.

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