Protein kinase-mediated phosphorylation of a purified sterol ester hydrolase from bovine adrenal cortex

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Abstract Sterol ester hydrolase (cholesterol esterase, E.C. 3.1.1.13) of bovine adrenal cortex has been extensively purified by ammonium sulfate fractionation, acid precipitation, hydroxylapatite chromatography, and Sephadex G-200 chromatography. During the purification sequence, the hydrolase activity was purified free of endogenous protein kinase. With this purified preparation, activation by cyclic AMP and ATP-Mg^{2+} did not occur unless exogenous protein kinase was included in the activating system. Using [γ-^{32}P]ATP, the transfer of the terminal phosphate to the enzyme protein was demonstrated by three separate experimental approaches. With pooled fractions from Sephadex G-200 chromatography, significant binding of ^{32}P by the enzyme protein was observed only in the presence of exogenous protein kinase. Time course studies disclosed a close concurrence between the extent of activation of the purified enzyme by cyclic AMP-dependent protein kinase and the level of ^{32}P transfer from [γ-^{32}P]ATP to the enzyme protein. Finally, assays carried out during Sephadex G-200 chromatography showed a correspondence in the peaks for activated sterol ester hydrolase and for ^{32}P binding by protein. The data confirm that the activation of adrenal sterol ester hydrolase by cyclic AMP and ATP-Mg^{2+} involves protein kinase-catalyzed phosphorylation of the enzyme protein.

Supplementary key words Cyclic AMP · ATP · [γ-^{32}P]ATP · enzyme phosphorylation

There are a variety of direct and indirect data which suggest that the enzymes of sterol ester metabolism in the adrenal cortex respond to hormonal stimuli (1, 2). Hypophysectomy in rats results in a decrease in adrenal cholesterol esterase activity (sterol ester hydrolase, E.C. 3.1.1.13) (3); in contrast, injection of ACTH into either normal (4) or hypophysectomized rats (3) causes an increase in the activity of this enzyme.

Davis (5) reported the first preliminary evidence for cyclic AMP stimulation of sterol ester hydrolase (SEH) activity in adrenal homogenates. Subsequently, a requirement for protein kinase in the activation process was reported for the enzyme in crude preparations of bovine adrenal cortex (6, 7). During partial purification of the enzyme, stimulation of SEH activity by cyclic AMP and ATP was lost, and was demonstrable only by readdition of protein kinase, together with the nucleotide cofactors (6, 7). Trzeciak and Boyd (7) also conducted studies with [γ-^{32}P]ATP using the 105,000 *g* supernatant of bovine cortical homogenates; transfer of ^{32}P label to the crude protein fraction was construed as evidence for SEH phosphorylation. However, this fraction contains a variety of potential protein receptors for ^{32}P, including protein kinase itself (8). During the preparation of the present report, Beckett and Boyd (9) reported on the partial purification (53-fold) of SEH from bovine adrenal cortex. This preparation was free of endogenous protein kinase but contained two protein bands on polyacrylamide disc electrophoresis. One of these proteins contained SEH activity and contained ^{32}P radioactivity after incubations with [γ-^{32}P]ATP. Also, the time course of activation of SEH closely paralleled that of phosphorylation of the enzyme.

In the present study, the hormone-sensitive SEH of bovine adrenal cortex was characterized and extensively purified (600-fold). During the course of purification, the enzyme was freed of endogenous protein kinase. With this preparation, an absolute requirement for protein kinase in the activation process was demonstrated and direct evidence for phosphorylation of the purified enzyme was obtained. A portion of these studies has been presented in a preliminary report (10).

Abbreviations: SEH, sterol ester hydrolase; TCA, trichloroacetic acid.

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EXPERIMENTAL PROCEDURES

Materials

[4-^{14}C]Cholesteryl oleate was obtained from New England Nuclear Corp., Boston, MA, or was prepared according to Swell and Treadwell (11). Purity was verified by thin-layer chromatography and by gas–liquid radiochromatography of the free cholesterol after alkaline hydrolysis and extraction. Carrier cholesteryl oleate (99%), ATP, cyclic AMP, and dibutyryl cyclic AMP were purchased from Sigma Chemical Co., St. Louis, MO. Protein kinase, prepared by the method of Gilman (12), and calf thymus histone (Type IIA) were also obtained from Sigma Chemical Co. The sodium salt of [γ-^{32}P]ATP was obtained from Amersham/Searle Corp., Arlington Heights, IL.

Tissue preparation

Bovine adrenal glands were obtained fresh from a local slaughterhouse and transported on crushed ice. The medulla was removed and the cortex was decapsulated and homogenized in 0.1 M phosphate buffer, pH 7.5, or phosphate buffer–0.25 M sucrose, pH 7.5. The homogenate was centrifuged for 1 hr at 105,000 *g*; the floating lipid layer was removed and the clear supernatant fraction (S_{105} fraction) was used as the crude enzyme source for further studies. When used directly in experiments, the S_{105} fraction was passed through Sephadex G-25 to remove the low molecular weight cofactors (13).

Assay of sterol ester hydrolase

SEH activity was determined by the release of [4-^{14}C]cholesterol from [4-^{14}C]cholesteryl oleate using a micro thin-layer chromatographic assay (14). Additions, as indicated in the text, were made to the enzyme preparation contained in 100 mM phosphate buffer, pH 7.4, in a final volume of 2.0 ml. The reaction was initiated by forceful addition of [4-^{14}C]cholesteryl oleate (0.15 μmol) in 100 μl of acetone (15). Incubations were at 37°C in a metabolic shaker. Aliquots (100 μl) were removed at 15-min intervals and extracted in 1.0 ml of acetone–ethanol 1:1. Separation of [4-^{14}C]cholesterol from [4-^{14}C]cholesteryl oleate was accomplished on microchromatoplates (14). Initial reaction rates were determined and enzyme activity was expressed as units, where 1 unit represents the release of 1 nmol free cholesterol/hr under the conditions of the assay. Enzyme activity was linearly related to enzyme concentration up to 9.0 mg/ml of the 105,000 *g* supernatant fraction protein. The assay was also linear with time up to 120 min at 37°C using 1 ml of supernatant fraction (9.0 mg protein/ml).

Purification of sterol ester hydrolase

Ammonium sulfate fractionation. The 105,000 *g* supernatant from bovine cortical homogenates was subjected to ammonium sulfate fractionation at 5°C. Protein precipitating at 25% saturation after 30 min was centrifuged down and discarded. Protein sedimenting at 40% saturation was obtained by centrifugation and redissolved in 10 mM phosphate buffer, pH 7.5. This was dialyzed against three changes of 10 mM phosphate buffer, pH 5.2, or was desalted by Sephadex G-75 chromatography. The gel was equilibrated with 10 mM buffer and elution was carried out at a flow rate of 15 ml/hr with the same buffer.

Acid precipitation. Pooled fractions from Sephadex G-75 chromatography were adjusted to pH 5.2 with 0.1 N acetic acid and kept on ice for 30 min with stirring. The protein precipitate was centrifuged at 15,000 *g* for 30 min and redissolved in 10 mM phosphate buffer.

Hydroxylapatite chromatography. Biogel HT (Bio-Rad Laboratories, Richmond, CA) was washed five times with 10 mM phosphate buffer prior to preparation of the column (2.5 × 45 cm). A flow rate of 10–15 ml/hr was maintained with an LKB Perplex pump. The acid-precipitated fraction was applied and elution was carried out with a linear gradient of 10 mM to 400 mM phosphate buffer, pH 7.5. Fractions containing sterol ester hydrolase activity were pooled and concentrated to 10 ml using a Diaflo apparatus with a PM-10 membrane (Amicon Corp., Lexington, MA).

Sephadex G-200 chromatography. Sephadex G-200, swollen in boiling distilled water for 5 hr, was equilibrated with 10 mM phosphate buffer, pH 7.5, at room temperature. A column (2.5 × 100 cm) was prepared with the degassed gel at 4°C and a flow rate of 10 ml/hr was established using 10 mM buffer. The column void volume was determined with 0.2% dextran blue and the column was standardized using 50 mg each of ribonuclease, ovalbumin, chymotrypsinogen, and aldolase.

The concentrated enzyme fraction from hydroxylapatite chromatography was applied and elution was carried out with 10 mM phosphate buffer, pH 7.5.

Protein kinase activity. Protein kinase activity was determined by the method of Jard and Bastidi (16). The assay system (2 ml) consisted of 0.1 mM $MgCl_2$, 1 μM cyclic AMP, 50 μM sodium [γ-^{32}P]ATP (1 μCi), 40 mg of histone, Type IIA, and the enzyme

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preparations in 10 mM phosphate buffer, pH 7.5. Incubations were carried out in air for periods up to 120 min at 37°C. Protein-bound ^{32}P was determined by precipitating protein in 25% trichloroacetic acid (TCA) containing 1 mM ATP. The mixture was filtered on Millipore 25 mm HAWP; protein was washed with 50 ml of 5% TCA and radioactivity was determined by scintillation spectrometry (Beckman LS-250).

Alternately, the TCA-insoluble protein was sedimented at 15,000 *g* for 20 min. The supernatant was discarded and the protein was solubilized in 2 ml of 0.5 M NaOH. The protein was reprecipitated with 10% TCA and recentrifuged two additional times prior to determination of radioactivity in 10 ml of Aquasol (New England Nuclear Corp.). The results from both procedures were comparable (**Fig. 1**) and reaction rates were linear with incubation time and with protein concentration. Protein kinase activity is expressed as pmol protein-bound ^{32}P/min.

Enzyme phosphorylation. Studies on the binding of ^{32}P from [γ-^{32}P]ATP by sterol ester hydrolase were conducted under the same conditions as described for protein kinase assays except that histone was omitted from the reaction medium. Enzyme-bound ^{32}P was assayed using the Millipore filtration technique.

Other methods. Free and esterified cholesterol were determined as the digitonides by the method of Sperry and Webb (17). Protein in the chromatographic fractions was estimated by absorbance at 280 nm on a Beckman Acta III spectrophotometer, and quantitative data were obtained by the method of Lowry et al. (18) using bovine serum albumin as a standard.

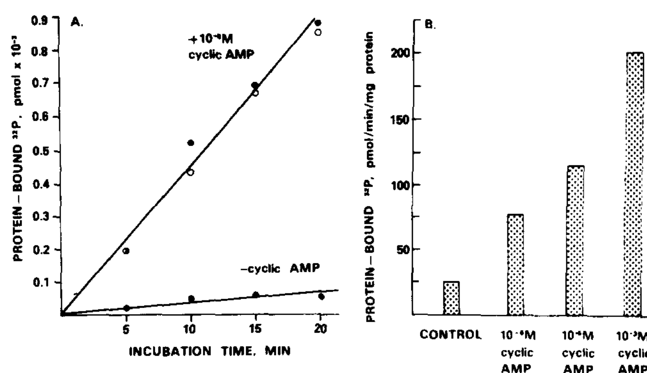

Fig. 1. Cyclic AMP-dependent protein kinase activity in S_{105} fraction of bovine adrenal. Left panel, *A*, indicates linearity of the assay in the absence and presence of 10^{-6}M cyclic AMP. Protein-bound ^{32}P was determined on TCA-precipitable protein after Millipore 25 mm HAWP filtration (closed circles), or after repeated precipitation with trichloroacetic acid (open circles). The right panel, *B*, indicates the effect of increasing concentrations of cyclic AMP on the transfer of ^{32}P from [γ-^{32}P]ATP (1 μCi) during incubations containing 1 mM $MgCl_2$, 50 μM ATP, 40 mg of histone, Type IIA, and the SEH preparation (S_{105} fraction) in 10 mM phosphate buffer, pH 7.5.

RESULTS

Comparison of substrates

Three [4-^{14}C]cholesteryl oleate (0.15 μmol) preparations were tested as substrates for adrenal SEH activity. These included the forceful addition of substrate in 100 μl of acetone to the enzyme preparation, as described earlier, an albumin-dispersed ester, and the ester in phospholipid–bile salt liposomes (15). The albumin-stabilized dispersion was hydrolyzed to only 18% of the level seen using substrate addition in acetone; the liposomal substrate was only 45% as effective as substrate added in acetone. Furthermore, using either albumin-stabilized or liposomal substrate preparations, it was not possible to detect any effect on enzyme activity by additions of various combinations of ATP, Mg^{2+}, and cyclic AMP. Therefore, all further studies on enzyme activation were conducted with the substrate in acetone added directly to the enzyme preparation.

Characteristics of the activation system

Sterol ester hydrolase showed a broad pH activity curve with a maximum between pH 7.0 and pH 7.5. However, in the presence of cyclic AMP, ATP, and Mg^{2+}, the shape of the pH activity curve differed, showing a sharp maximum at pH 7.5. Cofactor-independent sterol ester hydrolase activity (baseline activity) was always detected in the S_{105} fraction. When the baseline activity was low, activation by added cofactors was routinely observed. However, with higher baseline levels of activity, the extent of activation by cyclic AMP, ATP, Mg^{2+}, and protein kinase was either low or not detectable. With preparations containing high baseline SEH activity, partial deactivation of the enzyme was accomplished by Sephadex G-25 chromatography (13), or by incubation with Mg^{2+}-dependent phosphatase (0.14 units where 1 unit hydrolyzes 1 μmol of *p*-nitrophenylphosphate/min) for 2 hr at 37°C. As shown in **Fig. 2,** deactivation by both procedures was equivalent and enzyme activity was restored to, or near, its original level by incubations with cyclic AMP, ATP, and Mg^{2+}.

Direct evidence for the presence of cyclic AMP-dependent protein kinase in the S_{105} fraction of adrenal is shown in the right panel of Fig. 1.

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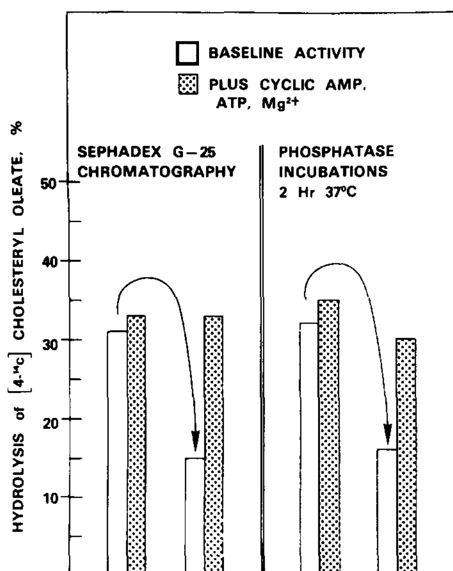

Fig. 2. Deactivation of baseline SEH activity in the S_{105} fraction by Sephadex G-25 chromatography or incubation with Mg^{2+}-dependent phosphatase. The S_{105} fraction was incubated with cyclic AMP, ATP, and Mg^{2+} before or after chromatography on Sephadex G-25 (left panel). For the data in the right panel, the S_{105} fraction was incubated with 0.14 units of Mg^{2+}-dependent phosphatase (1 unit hydrolyzed 1 μmol *p*-nitrophenylphosphate/min) for 2 hr at 37°C.

Transfer of ^{32}P from [γ-^{32}P]ATP to histone occurred at a low level in the absence of cyclic AMP; in contrast, ^{32}P transfer was stimulated 3-fold in the presence of 10^{-6}M cyclic AMP and was further increased with increasing cyclic nucleotide concentrations.

Evidence for a direct role of protein kinase in activation of SEH was obtained with a partially purified preparation of adrenal SEH (pH 5.2 precipitate, Table 1). As shown in **Fig. 3,** addition of cyclic AMP, ATP, and Mg^{2+} resulted in a 50% increase in enzyme activity from an initial level of 800 units/mg protein to 1200 units/mg protein. Further addition of 32, 64, and 96 μg of commercial rabbit muscle protein kinase resulted in a linear increase in enzyme activity. It was calculated that this enzyme preparation contained an amount of endogenous cyclic AMP-dependent protein kinase equivalent to 42 μg of commercial protein kinase. The purified enzyme preparation was used in further studies on the mechanism of this effect of protein kinase.

Purification of SEH and separation from endogenous protein kinase

The purification sequence for adrenal sterol ester hydrolase and the activity of endogenous protein kinase in each step of the purification are summarized in **Table 1.** In a preliminary study with the desalted ammonium sulfate fraction, it was found that incubation with 3 mM *p*-chloromercuribenzoate for 15 min resulted in 75% inhibition of SEH activity, and that this could be partially prevented by pretreatment with 20 mM dithiothreitol for 5 min prior to addition of *p*-chloromercuribenzoate. Thus, all buffers contained 1 mM dithiothreitol to protect sulfhydryl groups, and 0.2% (w/v) sodium azide to prevent bacterial contamination of the column packings.

When the desalted ammonium sulfate fraction was subjected to precipitation at pH 5.2 (13), approximately 80% of the sterol ester hydrolase activity and 50% of the protein kinase activity were recovered in the acid precipitate. This was redissolved in 10 mM phosphate buffer and subjected to hydroxylapatite chromatography, using a linear phosphate buffer elution gradient of 10–400 mM, pH 7.5. As shown in **Fig. 4,** enzyme activity was eluted as a single peak at 180–230 mM phosphate buffer. Recovery of enzyme activity ranged from 50 to 60% with a 3- to 5-fold increase in specific activity. In this step, approximately 98% of the protein kinase activity in the acid precipitate was eliminated from the pooled and concentrated active SEH fraction.

The final step of purification involved Sephadex

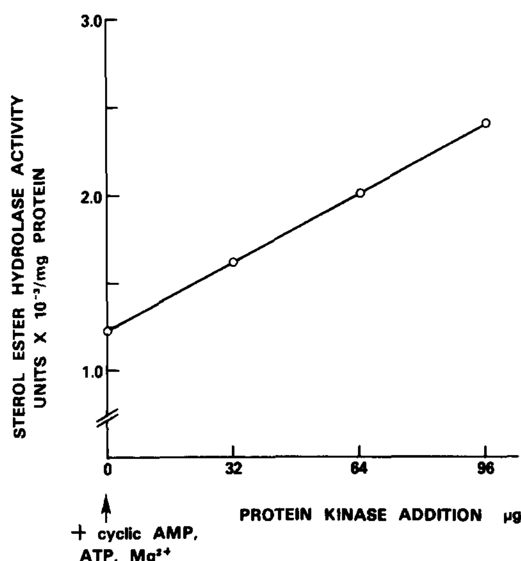

Fig. 3. Effect of addition of commercial rabbit muscle protein kinase on the activity of semi-purified preparation (pH 5.2 precipitate) of adrenal sterol ester hydrolase. The incubation media (2 ml) contained 1 ml of the enzyme preparation in 10 mM phosphate buffer, pH 7.5, 3 mM ATP, 10 mM Mg^{2+}, 20 μM dibutyryl cyclic AMP and 0.2 μCi of [4-^{14}C]cholesteryl oleate. Cyclic AMP-dependent protein kinase was added at the levels indicated and incubations were for 30 min at 37°C. Baseline activity prior to addition of cyclic AMP, ATP, and Mg^{2+} was 800 units/mg protein.

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G-200 chromatography; 10 mM phosphate buffer, pH 7.5, was used to equilibrate the column and for elution after charging of the column. Although recovery of the enzyme was only 20–25% in this step, the enzyme was eluted as a single peak with a purification factor of 6- to 10-fold and was completely devoid of protein kinase activity (Table 1). Overall recovery of total enzyme activity (combined baseline and activated enzyme) through the six purification steps was 6–10% with a purification of approximately 110-fold. The sterol ester hydrolase stimulated by cAMP was purified over 600-fold, based on the specific activities in Table 1.

Dependency of SEH activation of cyclic AMP-dependent protein kinase

The representative effect of cyclic AMP and ATP and of exogenous protein kinase on activation of SEH in the S_{105} fraction, in the pH 5.2 precipitate, and in the final Sephadex G-200 fraction is summarized in **Table 2.** There was a low level of activation in the S_{105} fraction, resulting from addition of cyclic AMP and ATP, and this activation was routinely more apparent as the purification proceeded. However, since the final enzyme preparation was devoid of endogenous protein kinase, the addition of only cyclic AMP and ATP to this fraction was consistently without effect on the activity of SEH. Conversely, added protein kinase was consistently without a marked effect when included in the activation system with crude SEH preparations. This finding is consistent with results obtained with the hormone-sensitive lipase of adipose tissue (13). As shown in Fig. 3 and Table 2, the stimulatory

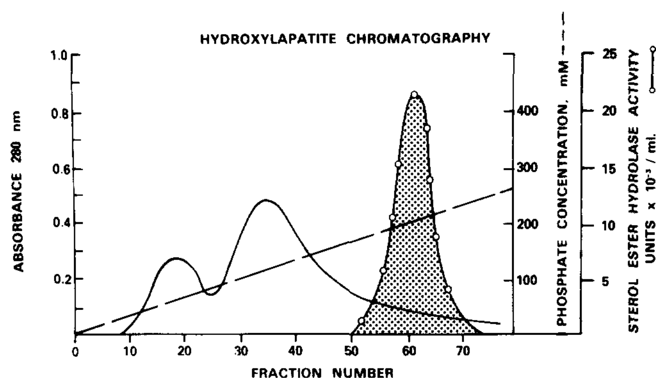

Fig. 4. Hydroxylapatite chromatography of adrenal sterol ester hydrolase. A column, 2.5 × 45 cm, was packed with Biogel HT equilibrated with 10 mM phosphate buffer, pH 7.5. The pooled and concentrated (Diaflo PM-10) from DEAE-cellulose chromatography was applied in 10 mM phosphate buffer. Elution was carried out with a linear gradient of phosphate buffer, pH 7.5, of from 10 to 400 mM.

effect of protein kinase on SEH activity could be consistently demonstrated when semi-purified preparations of the enzyme were employed. Finally, the absolute requirement for cyclic AMP-dependent protein kinase was demonstrable using the pooled and concentrated enzyme fraction from Sephadex G-200. With this enzyme preparation, addition of ATP and cyclic AMP, separately and together, had no effect on SEH activity. Similarly, addition of protein kinase, alone or with ATP, was without effect; addition of all three cofactors consistently resulted in marked activation of purified SEH (Table 2). These data are comparable to those reported by Beckett and Boyd (9).

TABLE 1. Summary of the purification of adrenal sterol ester

Steps	Sterol Ester Hydrolase: Total Enzyme Units[a] $\times 10^{-3}$	Sterol Ester Hydrolase: Specific Activity[b]	Protein Kinase Units[c] $\times 10^{-2}$
1. S_{105} fraction	457	170	319
2. Ammonium sulfate, 25–40% precipitate dialyzed	350	340	
3. Sephadex G/75	300	633	250
4. pH 5.2 precipitate	238	850	122
5. Hydroxylapatite chromatography	130	2,890	2.5
6. Sephadex G/200 chromatography	28	18,461	0

[a] Defined as nmol free cholesterol produced/hr using the conditions described under Experimental Procedures.
[b] Units/mg protein.
[c] Defined as pmol protein-bound ^{32}P/min using the conditions described under Experimental Procedures.

Protein kinase-dependent phosphorylation of purified sterol ester hydrolase

Phosphorylation of the purified SEH, and its relation to enzyme activation, was demonstrated by three techniques, two of which have been employed in similar studies with adipose tissue lipase (19, 20). In the first approach, aliquots of the pooled and concentrated active fractions from Sephadex G-200 chromatography were employed for determination of phosphate transfer from [γ-^{32}P]ATP, using the same conditions as for protein kinase assay (see Experimental Procedures) except that histone was omitted from the incubations. Purified SEH (200 μg) and protein kinase (100 μg) were included in a reaction mixture containing cyclic AMP, Mg^{2+}, and 6 μg of cholesteryl oleate (added in 100 μl of acetone). Reaction was initiated by addition of [γ-^{32}P]ATP and incubations were for 2 hr at 37°C. Incubations in which the enzyme and protein kinase were omitted were employed as controls. As shown

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TABLE 2. Activation of adrenal sterol ester hydrolase at various stages of purity

Additions to Control[a]	S_{105} Fraction: Specific Activity[b] $\times 10^{-3}$	S_{105} Fraction: % Activation	pH 5.2 Precipitate: Specific Activity $\times 10^{-3}$	pH 5.2 Precipitate: % Activation	Sephadex G-200: Specific Activity $\times 10^{-3}$	Sephadex G-200: % Activation
None	0.17		1.1		18.5	
Cyclic AMP (20 μM) ATP (3 mM)	0.20	18	1.5	36	18.5	0
Protein kinase (100 μgm)					18.5	0
Cyclic AMP, ATP, protein kinase	0.20	18	2.6	136	36.6	98

[a] Controls contained 2 ml of the appropriate enzyme fraction in 100 mM phosphate buffer, pH 7.4; 10 mM Mg^{2+}; and 0.15 μmol of [4-^{14}C]cholesteryl oleate added in 100 μl acetone.
[b] Expressed as nmol free cholesterol produced/hr/mg protein.

in **Fig. 5,** protein-bound ^{32}P in the presence of both enzyme and cyclic AMP-dependent protein kinase was approximately 2250 pmol, while in the absence of enzyme, TCA-precipitable ^{32}P was negligible. The low level of ^{32}P-binding in the absence of protein kinase was a consistent finding; this is probably nonspecific binding to either SEH or contaminating protein since, under these conditions, ^{32}P binding was not associated with enzyme activation (see Table 2).

In a second approach, the time course of protein phosphorylation and enzyme activation was determined and is shown in **Fig. 6.** Sampling times were based on preliminary studies indicating a relatively slow enzyme activation by cyclic AMP, ATP, and protein kinase. It is readily apparent that there is good correspondence between the rate of activation of purified SEH and the amount of TCA-precipitable ^{32}P. These data are comparable to those obtained with adipose tissue lipase (19, 20) and adrenal sterol ester hydrolase (9).

A third evidence for the relation between protein kinase-dependent activation and phosphorylation of the adrenal SEH was obtained by measurement of enzyme activation and protein phosphorylation in the absence of added histone ^{32}P receptor during the course of Sephadex G-200 chromatography of the purified enzyme fraction. One-ml aliquots of the fractions during chromatography were used for the following assays: (*a*) baseline SEH activity; (*b*) SEH activity in the presence of cyclic AMP, ATP, and protein kinase; (*c*) TCA-precipitable ^{32}P in the absence of added protein kinase; (*d*) TCA-precipitable ^{32}P catalyzed by added cyclic AMP-dependent protein kinase; and (*e*) endogenous protein kinase activity using histone receptor. The data summarized in **Fig. 7,** show the correspondence in the peaks for activated sterol ester hydrolase and protein kinase-dependent phosphorylation (absence of histone receptor). This enzyme activity corresponds to a molecular weight of approximately 350,000, which is dissimilar to the estimated molecular weight (204,000) reported by Beckett and Boyd (9), but is similar to

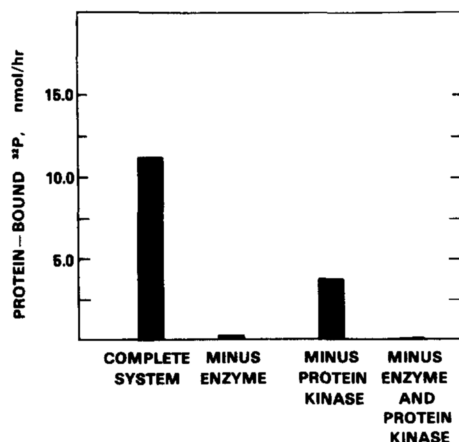

Fig. 5. Transfer of the terminal phosphate of [γ-^{32}P]ATP to protein in the presence and absence of purified adrenal SEH and cyclic AMP-dependent protein kinase. The complete system contained: 200 μg of purified SEH (Sephadex G-200 fraction) in 10 mM phosphate buffer, pH 7.5; 100 μg of protein kinase; 0.1 mM $MgCl_2$; 1 μM cyclic AMP; and 50 μM sodium [γ-^{32}P]ATP (1 μCi). Incorporation of ^{32}P into TCA-precipitable protein was determined after 2-hr incubations at 37°C.

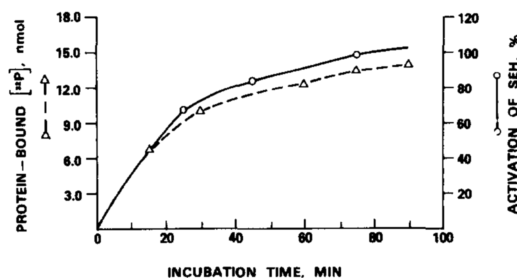

Fig. 6. Time course of protein kinase-mediated activation of purified adrenal SEH and transfer of the terminal phosphate of [γ-^{32}P]ATP to enzyme protein. The incubation media contained 200 μg of purified SEH in 10 mM phosphate buffer, pH 7.5; 100 μg of protein kinase; 0.1 mM $MgCl_2$; 1 μM cyclic AMP; and 50 μM sodium [γ-^{32}P]ATP (1 μCi). Aliquots were removed at the times indicated for determination of SEH activity and incorporation of ^{32}P into TCA-precipitable protein.

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the estimated molecular weight (400,000) of purified sterol ester hydrolase of rat pancreas (21) and pancreatic juice (22). The reason for this discrepancy is not clear.

The separation of endogenous protein kinase activity (determined in the absence of added protein kinase and the presence of histone) from the major peaks of enzyme activation and phosphorylation is also shown in Fig. 7. From the position of this peak, the molecular weight of endogenous adrenal protein kinase was estimated to be approximately 170,000. This agrees reasonably well with the reported minimal value (152,000) of cyclic AMP-dependent protein kinase of adrenal cortex (23).

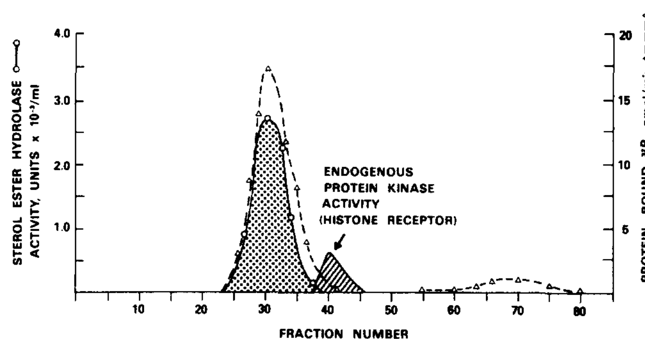

Fig. 7. Elution profiles of adrenal SEH activity, incorporation of ^{32}P into TCA-precipitable protein and endogenous protein kinase activity during Sephadex G-200 chromatography. Endogenous protein kinase activity was determined in the presence of the histone ^{32}P receptor during incubations with [γ-^{32}P]ATP (16). Incorporation of ^{32}P into TCA-precipitable protein in the absence of added histone (△ - - - △) indicates phosphorylation of the purified SEH and corresponds to the elution of SEH activity.

DISCUSSION

It is well recognized that steroidogenesis in adrenal cortex and similar steroid-elaborating tissues is under complex and multisite hormonal control (1, 2, 23, 24); the rate-limiting step in the pathway appears to be the utilization of unesterified cholesterol for mitochondrial pregnenolone synthesis. Other hormone-sensitive sites include microsomal 17-hydroxylation, ribosomal protein synthesis, whose role is yet to be defined, and hydrolysis of the sterol esters contained in cytoplasmic inclusion droplets.

Available evidence suggests that endogenous sterol esters represent an important storage form of the cholesterol precursor for sustained steroidogenesis (2). Thus, during a variety of stress conditions in animals (25, 26), or following injection of ACTH over several hours (3, 27), the sterol esters are gradually hydrolyzed, resulting in a measurable disappearance of the numerous lipid droplets in the cortical tissue (28). Similarly, in vitro, low levels of ACTH (5 μunits) will elicit a steroidogenic response by adrenal cells which is dependent on utilization of available free cholesterol in an active precursor pool (23), since under these conditions, hydrolysis of endogenous sterol esters is not detectable.[2] This steroidogenic effect is, however, short-lived due, perhaps in part, to endocytosis and subsequent lysosomal degradation of the peptide hormone (29). When higher levels of ACTH are used, or when cells are continuously exposed to ACTH, continued steroidogenesis is dependent on a supply of sterol precursor. Since cholesterolgenesis under these conditions appears negligible (30), the major source of the substrate must be derived from sustained hydrolysis of the sterol esters. This latter process, catalyzed by sterol ester hydrolase, is not a secondary response to depletion of the active free cholesterol pool, but is itself under direct hormonal regulation (6, 7). Thus, changes in the specific activity of sterol ester hydrolase in vivo are correlated directly with the availability of circulating ACTH (3, 4), and enzyme activation in vitro is dependent on cyclic AMP and protein kinase (6, 7).

The present study provides definitive information on the mechanism of activation of this hormone-sensitive sterol ester hydrolase of adrenal cortex. It has been shown that the inability to determine a direct requirement for protein kinase in crude homogenates or the S_{105} fraction of adrenal is due to a high level of endogenous protein kinase in this tissue, which has a cellular distribution similar to that of the hydrolase (8). However, a complete dependency of SEH activation on cyclic AMP-dependent protein kinase has been demonstrable with a partially purified enzyme freed of measurable endogenous kinase activity. In the absence of cyclic AMP, there is no activation, and only low control levels of phosphate transfer are apparent. In the presence of cyclic AMP, ATP, and rabbit muscle protein kinase, maximal activation of the enzyme is apparent. This activation of SEH is accompanied by a transfer of the terminal phosphate of ATP to the enzyme protein. Furthermore, there is an excellent correlation (Fig. 6) between the level of enzyme activation and the extent of phosphate transfer to the enzyme protein. These rates of activation and phosphorylation are somewhat slower than those reported for adipose tissue lipase (19) and adrenal sterol ester hydrolase (9), but are compatible with the apparent

[2] Vahouny, G. V., R. Chanderbhan, and G. A. Hodges. Unpublished data.

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rates of sterol ester hydrolysis observed in vivo (25) or in adrenal cells exposed to ACTH[3] (26). Corticosterone release by adrenal cortical cells is detected within 24 sec after exposure to ACTH (31). Thus, the activation of sterol ester hydrolase by cyclic AMP-dependent protein kinase is not a rate-limiting process in steroidogenesis in this tissue, but rather may represent an important control on the availability of precursor for continued steroidogenic capacity.

Preliminary evidence for reversible deactivation of SEH has also been obtained. First of all, it is highly likely that factitious activation of SEH occurs during the obtaining and handling and/or the homogenization of the tissue. Similar conclusions have been reached with the SEH of bovine corpus luteum[3] and with chicken adipose tissue lipase (13). In order to obtain a crude lipase preparation responsive to activation by cofactors, it had been necessary to preincubate the tissue preparation for 3–4 hr prior to study (13). Alternately, deactivation of hormone-sensitive lipase has been accomplished by dialysis at 4°C. This deactivation was shown to be Mg^{2+}-dependent (9) and is suggestive of the presence of a Mg^{2+}-dependent phosphatase (13). With the adrenal SEH, deactivation in crude preparations was accomplished by preincubation of the tissue homogenate for 2 hr at 37°C (6), or by addition of Mg^{2+}-dependent phosphatase. This latter finding provides more direct evidence that deactivation of active SEH may involve dephosphorylation of the activated enzyme. Evidence for a direct correlation of phosphorylation–dephosphorylation with parallel changes in enzyme activity has recently been provided (9). In this regard, Severson, Khoo, and Steinberg (32) have shown that the SEH and lipase activities of chicken adipose tissue are probably deactivated by an endogenous phosphoprotein phosphatase. This conclusion was based on the findings that reversible deactivation is enzymatic (heat-sensitive), is reversed by incubations with ATP, Mg^{2+}, and cyclic AMP, and can be accomplished by addition of exogenous protein phosphatase (32).

In conclusion, the present studies have confirmed the presence of a hormone-sensitive cholesterol esterase in bovine adrenal cortex, and have provided direct evidence for protein phosphorylation as the mechanism of protein kinase-dependent activation. There is not yet any evidence as to the sites of protein phosphorylation, or on the mechanism(s) of deactivation of the enzyme. In addition, it remains to be determined whether similar increases in enzyme activity in the adrenal cortex can be correlated directly with the depletion of adrenal sterol ester associated with stress or after injection of ACTH.

[3] Bisgaier, C., and G. V. Vahouny. Unpublished data.

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