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Cholesterol side-chain cleavage in the rat adrenal cortex: Isolation of a cycloheximide-sensitive activator peptide

(corticotropin/peptide HPLC/steroidogenesis)

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ABSTRACT **A cytosolic peptide activator (M_r ≈2,200) of cholesterol side-chain cleavage in the adrenal cortex has been isolated from normal corticotropin-treated rats and from rats implanted with the MtT/F4 corticotropin-secreting pituitary tumor. The isolation techniques were those common to peptide hormone purification, including tissue extraction into a highly acidic medium, gel filtration, and reverse-phase HPLC. The amino acid composition has been determined on acid hydrolysates. The activity of this adrenal peptide is acutely increased in hypophysectomized animals treated with corticotropin, and this increase is blocked by cycloheximide. The addition of activator peptide to adrenal mitochondrial preparations results in a rapid stimulation of pregnenolone formation that is dependent on activator concentration and a source of NADPH. In the absence of NADPH, addition of activator peptide to adrenal mitochondria increases the rate of cholesterol association with side-chain cleavage cytochrome P-450. The peptide therefore exhibits properties that are believed to characterize the hypothetical corticotropin-dependent labile activator of adrenal steroidogenesis.**

Corticotropin (ACTH) promotes an acute, cAMP-mediated (1) increase in the rate of cholesterol association with the mitochondrial side-chain cleavage (scc) cytochrome P-450 (cytochrome P-450$_{scc}$) in the adrenal cortex (2–4). This association is a major regulatory point in the steroidogenic pathway, resulting in the conversion of cholesterol to pregnenolone (5, 6). Furthermore, an ACTH-regulated protein may be an important intermediate at this step in steroid formation. This is inferred from studies indicating that various inhibitors of protein synthesis (2, 7–10), including cycloheximide, can prevent or reverse ACTH stimulation of the adrenal. Although cycloheximide does not block the mitochondrial accumulation of cholesterol in response to ACTH (11), it effects a decreased association of cholesterol with the enzyme's active site (12). Moreover, because the adrenal response to ACTH is expressed so rapidly (13), a mechanism involving ACTH-mediated activation of a labile protein, rather than *de novo* protein synthesis, has been proposed (8, 9, 14).

Although there have been several attempts to characterize this hypothetical modulator (15–20), none has been fully successful. Recently, experiments have been published (21) which suggest that the factor may have the properties of a basic polypeptide. We therefore approached the problem of isolation by applying techniques that have proven successful in purifying basic hydrophobic hormonal peptides. Bioactivity was monitored with a sensitive *in vitro* assay for cholesterol scc activity.

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MATERIALS AND METHODS

Animal Experimentation. Rats (160–180 g) were individually caged in rooms with controlled temperature (20 ± 1°C) and illumination (0600–1800 hr). Rat chow and water were allowed ad lib. Sprague–Dawley females (Holtzman) were the source for adrenal activator peptide in early isolation experiments and for the adrenocortical mitochondria used in the cholesterol scc assay. Where indicated, Fischer-344 females (Charles River Breeding Laboratories) were implanted (22) with the ACTH-secreting MtT/F4 mammotropic pituitary tumor (23) and maintained for 3–5 wk before they were used in experiments.

Isolation of Activator Peptide. A 105,000 × *g* supernatant fraction was prepared from rat adrenals (40 pair per experiment) as described (24). This was mixed with an equal volume of chilled peptide extraction medium (25) [initial concentrations: 2 M HCl, 10% (vol/vol) formic acid, 2% trifluoroacetic acid (Pierce), and 20 mg of NaCl per ml]. After standing for 30 min at 4°C, the solution was centrifuged (5,000 × *g*, 30 min). The supernatant was defatted successively with 5 vol each of isooctane and methylene chloride and then lyophilized. Vessels of siliconized glass or polypropylene were used for all peptide manipulations.

In subsequent studies entailing HPLC purification, several modifications were made in this protocol. First, adrenals were homogenized directly in extraction medium (1 M HCl/5% formic acid/10% trifluoroacetic acid containing NaCl at 10 mg/ml). Second, the defatted extract was chromatographed on a cartridge of C_{18}-silica (SepPak, Waters Associates) preequilibrated with acetonitrile and 0.1% aqueous trifluoroacetic acid. The cartridge was eluted sequentially with 10 ml of the aqueous solvent, 5 ml of 25% acetonitrile in aqueous solvent, and 3 ml of 60% acetonitrile in aqueous solvent. The final 3-ml fraction was lyophilized before reverse-phase HPLC (Varian model 5000 chromatograph coupled in series to a Varian Vari-Chrom spectrophotometer and a Perkin–Elmer model 204 spectrofluorometer). A MicroPak-SP C_{18} column (0.4 × 15 cm, 3-μm packing; Varian) was used for peptide and amino acid separations.

Activator Peptide Assay. Adrenal mitochondria were isolated (24) from rats pretreated with cycloheximide and ACTH (see Table 1), ensuring preparations with low basal cholesterol scc activity (≈0.2 nmol of pregnenolone per min per mg of protein) but increased concentrations of cholesterol substrate (11). Washed mitochondria were resuspended in enzyme reaction buffer (24) (0.25 mg of protein per ml), distributed (200-μl portions) into polypropylene tubes, and warmed for 10 min at 37°C. Lyophilized chromatographic fractions, each resuspended in 50 μl of the same buffer, were added and the preincubation was continued for 5 min. Control aliquots of the appropriate chro-

Abbreviations: ACTH, corticotropin; scc, side-chain cleavage.

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matographic mobile phase were treated identically. The cholesterol scc reaction was then carried out for 2 min as described (24). Pregnenolone product was extracted into methylene chloride and quantitated by specific radioimmunoassay (26). The pregnenolone in zero-time control incubations (routinely ≤0.03 nmol/mg of protein) has been subtracted from all assay values.

Amino Acid Analysis. Aliquots of HPLC-purified peptide were hydrolyzed in 0.20 ml of HCl/trifluoroacetic acid (2:1, vol/vol; Pierce) (27). Phenol (4 μg per tube) and an internal standard (500 pmol of ethanolamine per tube; Sigma) were included. The procedure of Umagat *et al.* (28) was used for the reaction of the dried hydrolysates with *o*-phthaldialdehyde (Sigma) and to separate the derivatized amino acids by HPLC with fluorescence detection. The solvent gradient was run exactly as described (28), but the flow rate was decreased to 1.0 ml/min to accommodate the higher back pressure of our column. Cysteine was determined as cysteic acid after sample peroxidation and hydrolysis (28). Proline content was assessed after reaction of an aliquot of hydrolysate with 4-chloro-7-nitrobenzofurazan (Sigma) (28). The absence of tryptophan was confirmed fluorometrically.

Miscellaneous. An Aminco DW-2 recording spectrophotometer was used to measure the pregnenolone-induced type II absorbance change (24) in preparations of adrenal mitochondria from hypophysectomized rats. The cytochrome P-450 concentration was determined according to Omura and Sato (29). Cholesterol was measured enzymatically with coupled fluorescence detection (30). Protein concentrations were determined by the method of Bradford (31).

RESULTS

Preliminary studies were designed to isolate a crude, nonlipoidal fraction from adrenocortical cytosol that would stimulate mitochondrial cholesterol scc *in vitro*. For this purpose, the 105,000 × *g* supernatant from adrenal homogenates of ACTH-stimulated rats was extracted, defatted, and chromatographed on Sephadex G-50 under acid-denaturing conditions. Each chromatographic fraction was lyophilized and assayed for its effect on cholesterol scc activity. The adrenal mitochondria used in this assay were prepared from a second group of animals that had been pretreated with cycloheximide and ACTH (we have also carried out the assay successfully with adrenal mitochondria from hypophysectomized rats).

A major peak of enzyme-stimulating activity was observed in cytosolic fractions 45–55 (Fig. 1). Cholesterol and pregnenolone were virtually undetectable in these fractions. On the other hand, fractions eluting in the void volume contained material that inhibited scc activity. This has been reported (15, 19) and was not investigated further. To obtain a better estimate of the molecular weight of the stimulatory material, adrenal cytosol from ACTH-stimulated animals was chromatographed on Sephadex G-25 (Fig. 2). A small peak (I) of stimulatory activity eluted near the void volume (fractions 27–32) and a larger peak (II) of activity was observed in fractions 50–56 (apparent $M_r \approx 2,200$).

When donor animals were pretreated with both cycloheximide and ACTH, their adrenal cytosol had diminished enzyme-stimulating activity associated with peak I fractions and no detectable activity in the peak II region (dotted line in Fig. 2; Table 1). The adrenal cytosol prepared from animals hypophysectomized 24 hr beforehand had sharply lower levels of activity associated with both peaks. Intravenous replacement of ACTH in a second group of hypophysectomized rats at 10 min before decapitation blocked this decline. Even higher stimulating activity was associated with peak II when total adrenal homogenate, rather than the cytosolic subcellular fraction, was extracted and chromatographed. Peak I activity nearly disappeared.

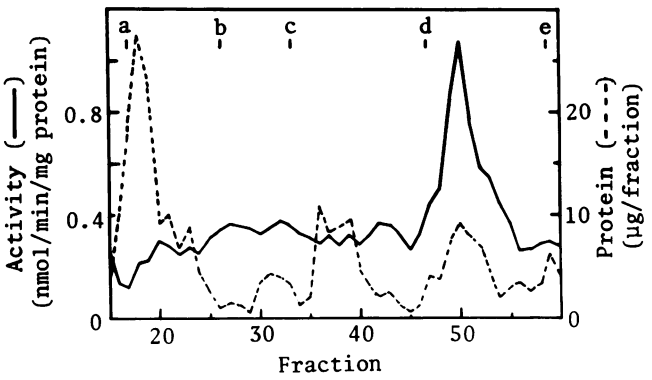

FIG. 1. Cholesterol scc-stimulating activity in Sephadex G-50 fractions of adrenal cytosol. A lyophilized acid extract of adrenal cytosol from 40 ACTH-treated rats was reconstituted in 0.2 ml of 0.2 M HCl and loaded onto a column (1 × 50 cm) of Sephadex G-50 superfine (Pharmacia). The column was eluted upward with 0.1 M HCl (2 ml/hr). Half of each 2-ml fraction was lyophilized and assayed for cholesterol scc stimulation (——); basal activity was 0.23 nmol of pregnenolone per min per mg of mitochondrial protein. The remainder was used for protein (---), cholesterol, and pregnenolone determinations. The column was precalibrated with void (a) and total (e) volume markers (blue dextran and dinitrophenylalanine, respectively) and the following standards (M_r): b, lima bean trypsin inhibitor, 9,195; c, aprotinin, 6,520; d, ACTH-(1–24), 2,934.

In order to demonstrate that the enzyme-stimulating activities were from the adrenal cortex, animals were subjected to bilateral adrenal enucleation and 8 wk of adrenocortical regeneration prior to tissue collection. Both peaks of activity were still apparent in the adrenal cytosol (Table 1). No activity was observed in Sephadex G-25 fractions collected from liver homogenates of ACTH-treated rats.

Because of its greater potency in stimulating cholesterol scc, further attempts at purification and characterization were focused on the material in peak II. In addition to the virtual absence of cholesterol and pregnenolone, no phospholipid was detected in this peak [assayed as total phosphorus (33)]. The stimulating activity was sensitive to preincubation with Pronase (79% decrease compared with a control preparation) but only slightly affected (12% decrease) by heat treatment (100°C, 2 min).

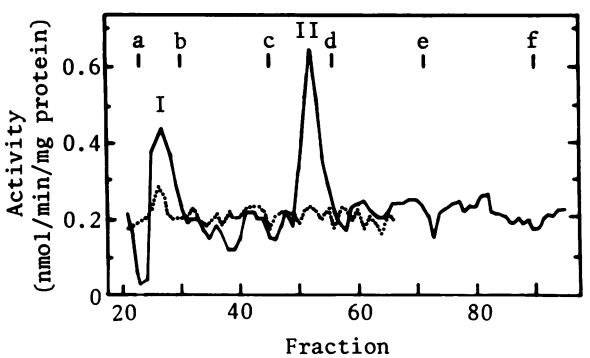

FIG. 2. Cholesterol scc-stimulating activity in Sephadex G-25 fractions of adrenal cytosol. Lyophilized acid extracts of adrenal cytosol from rats (40 per group) treated with ACTH (——) or with ACTH and cycloheximide (·····) were reconstituted in 0.2 ml of 0.2 M HCl and loaded onto a column (1.5 × 55 cm) of Sephadex G-25 superfine (Pharmacia). The column was eluted upward with 0.1 M HCl (3 ml/hr). Fractions (1.0 ml) were assayed for cholesterol scc stimulation (basal activity, 0.20 nmol of pregnenolone per min per mg of mitochondrial protein). Column void (a) and total (f) volume markers as in Fig. 1; other markers (M_r) were: b, ACTH-(1–39), 4,560; c, ACTH-(1–24), 2,934; d, γ-melanotropin-(1–14), 1,883; e, angiotensin II, 1,032.

Table 1. Stimulation of cholesterol scc activity by material in Sephadex G-25 peaks I and II

Group	Source of sample: Animal pretreatment	Source of sample: Extract	Cholesterol scc activity, nmol pregnenolone/min/mg protein: Peak I	Cholesterol scc activity, nmol pregnenolone/min/mg protein: Peak II
A	Hypox	Adrenal cytosol	0.20 ± 0.02	0.21 ± 0.02
B	Hypox + ACTH	Adrenal cytosol	0.38 ± 0.05*	0.71 ± 0.07*
C	ACTH	Adrenal cytosol	0.44 ± 0.03*	0.66 ± 0.05*
D	CH + ACTH	Adrenal cytosol	0.28 ± 0.01*	0.22 ± 0.02
E	ACTH	Adrenal whole homogenate	0.21 ± 0.03	0.82 ± 0.05*
F	ACTH	Adrenal cortex cytosol	0.29 ± 0.03*	0.69 ± 0.07*
G	ACTH	Liver whole homogenate	0.21 ± 0.01	0.19 ± 0.01

Hypophysectomies (Hypox) were performed 20–24 hr before experiments. Rats in groups C–G were killed with pituitaries intact. Cycloheximide (CH; Sigma) (15 mg per animal, intraperitoneal) and ACTH-(1–24) (Cortrosyn; Organon) (100 ng per animal, intravenous) were injected 30 min and 5 min, respectively, before decapitation. To eliminate medullary tissue (group F), animals were subjected to bilateral adrenal enucleation (32) and 8 wk of cortical regeneration. The 105,000 × *g* supernatant (cytosol; groups A, B, C, D, F) or whole homogenate (groups E, G) was prepared (40 adrenal pairs or 10 livers per experiment), extracted, and chromatographed on Sephadex G-25. No correction has been made for small differences between groups in the total amount of nonprecipitable protein chromatographed. Triplicate aliquots (0.30 ml) of each chromatographic fraction were lyophilized and tested *in vitro* for effect on cholesterol scc activity in adrenal mitochondria. Values (mean ± SD) for peak fractions I and II (Fig. 2) are given.
*$P < 0.05$ for difference from group A.

The material in Sephadex G-25 peak II fractions was subjected to further purification by reverse-phase peptide HPLC. Fractions were lyophilized and assayed for *in vitro* stimulating activity as before. A major peak of activity (arrow, Fig. 3) was partially resolved in one solvent system and then rechromatographed to apparent homogeneity in a second system (Fig. 4). This purified material was hydrolyzed and subjected to amino acid analysis. The results from determinations carried out on six separate samples were as follows (calculated mean value, nearest integer): Ala, 1.3, 1; Arg, 1.2, 1; Asx, 1.8, 2; Cys, 2.1, 2; Glx, 4.2, 4; Gly, 2.4, 2; His, 0.2, 0; Ile, 1.0, 1; Leu, 0.9, 1; Lys, 1.4, 1; Met, 0, 0; Phe, 0.8, 1; Pro, 0, 0; Ser, 1.7, 2; Thr, 0.3, 0; Trp, 0, 0; Tyr, 0.8, 1; Val, 1.1, 1. Exclusive of any potential structural features lost to analysis during acid hydrolysis, the composition indicates a M_r of 2,231.

Using quantitative amino acid analysis to correlate peptide mass with HPLC-associated UV absorbance (270 nm), we estimate the yield of activator peptide from ACTH-stimulated rats to range between 0.3 and 0.8 ng/mg wet weight of adrenal tissue. However, by implanting rats with the ACTH-secreting MtT/F4 pituitary tumor for 3–5 wk, the total content per gland of this adrenal peptide could be increased about 10-fold. (Because organ weight is also increased, this corresponds to a 2- to 3-fold increase per mg of tissue.) The properties of the activator peptide prepared from adrenals subjected to this chronic stimulation appear to be identical to those of the corresponding peptide isolated from healthy, acutely stimulated animals. This is noteworthy because, in addition to ACTH, the tumor also secretes large quantities of growth hormone and prolactin (23).

The intracellular location of adrenal activator peptide was assessed by direct HPLC quantitation. For this purpose, subcellular fractions from a pool of 60 adrenals were prepared and extracted. The results (Table 2) indicate that 92% of total peptide content recovered is with the 105,000 × *g* supernatant.

The addition of 1.0 μM HPLC-purified activator peptide to adrenal mitochondria stimulated a rapid increase in the customary biphasic rate of cholesterol scc (Fig. 5). The enzyme response (as determined at 2 min) exhibited a linear dependence on the concentration of added peptide (Fig. 6). Lineweaver–Burk analysis of these data (not shown) indicated apparent K_a and V_{max} for the activator of 0.3 μM and 0.9 nmol of pregnenolone per min per mg of protein, respectively. Stimulation of enzyme activity by the peptide was dependent on a generating system for reducing equivalents.

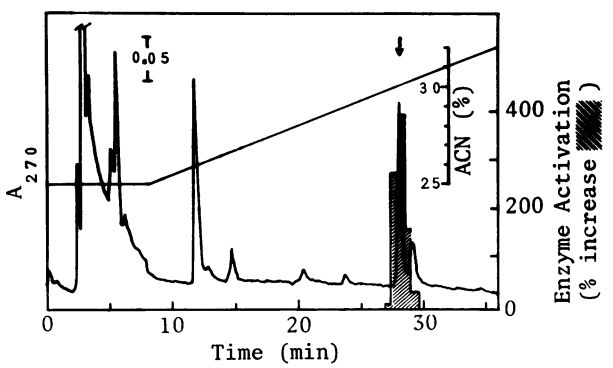

FIG. 3. HPLC separation of Sephadex G-25 peak II. Peak II cholesterol scc-stimulating fractions from Sephadex G-25 chromatography (Fig. 2) were lyophilized, reconstituted in 0.2 ml of aqueous buffer (0.25 M triethylammonium formate, pH 3.0), and subjected to reverse-phase HPLC (ambient temperature; flow, 0.5 ml/min). A gradient (0.25%/min) of 25–32% (vol/vol) acetonitrile (ACN) in the same buffer was used. Fractions (0.5 ml) were lyophilized and assayed for cholesterol scc stimulation (cross-hatching: percentage increase in activity over basal level of 0.19 nmol of pregnenolone per min per mg of mitochondrial protein).

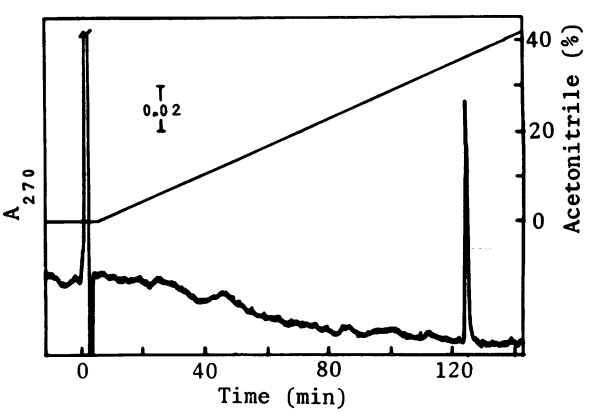

FIG. 4. HPLC rechromatography of the active peak in Fig. 3. The HPLC fractions that stimulated cholesterol scc (arrow, Fig. 3) were rechromatographed in a second solvent system (gradient 0–50% acetonitrile in 0.1% aqueous trifluoroacetic acid; 0.33%/min; 1.5 ml/min). A major absorption peak eluted at 36% acetonitrile and was bioactive.

Table 2. Activator peptide content of adrenal subcellular fractions

Fraction	Total content, ng/adrenal pair	Specific content, μg/mg of nonprecipitable protein
Whole homogenate	45.2	1.42
10,000 × *g* pellet	1.7	0.43
105,000 × *g* pellet	1.4	0.39
105,000 × *g* supernatant	37.1	1.54

Fractions were prepared by differential centrifugation (24) from 60 pairs of ACTH-stimulated rat adrenals and chromatographed in the HPLC system of Fig. 3. Peptide mass in each fraction was determined from its integrated absorbance peak (270 nm). Total protein was measured in the acid extract prior to chromatography.

Finally, 0.2 μM activator peptide effected a 3.7-fold increase in the pregnenolone-induced type II absorbance change of cytochrome P-450$_{scc}$ when added to adrenal mitochondria from hypophysectomized rats ($\Delta A_{390-420}$/mM cytochrome P-450: control, 8.0; peptide added, 29.4). The magnitude of the absorbance change reflects the amount of cholesterol associated with the enzyme complex. These results are therefore interpreted to indicate that activator peptide appears to stimulate this substrate–enzyme association. The increase is comparable to that observed with mitochondria from ACTH-treated (2) or stressed (34) animals.

DISCUSSION

We have successfully isolated an adrenal peptide activator of cholesterol scc by using techniques chosen with its hypothetical properties in mind. In particular, adrenal tissue was collected at 5 min after stimulation, when cholesterol scc activity is maximal (35). Moreover, the isolation procedure was designed to minimize artifactual proteolysis and adsorption to glassware. Advantage was also taken of the high resolving power of reverse-phase peptide HPLC.

The adrenal level of peptide activity (Table 1) increases acutely in response to ACTH, decreases in its absence (hypophysectomy), and is sensitive to cycloheximide. Although we customarily extracted whole adrenals, the presence of substantial activity in demedullated glands suggests that it is localized in the cortex (Table 1). At the subcellular level, nearly all of the peptide was isolated from the 105,000 × *g* supernatant fraction (Table 2). However, this could be a preparative artifact and does not preclude the possibility that a noncovalent association with the mitochondrial membrane exists *in vivo*. No comparable activity was observed in homogenates of rat liver (Table 1), an organ containing several cytochrome P-450 enzyme systems. Furthermore, HPLC separation of a liver extract did not produce a detectable peak with the appropriate retention time (data not shown).

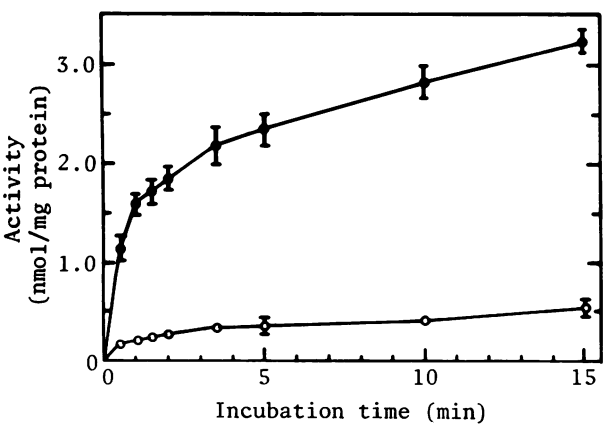

FIG. 5. Time course of pregnenolone formation by adrenal mitochondria incubated with (●) or without (○) 1.0 μM activator peptide. Values reflect mean ± SD of triplicate incubations. Absence of error bars indicates an error less than symbol height.

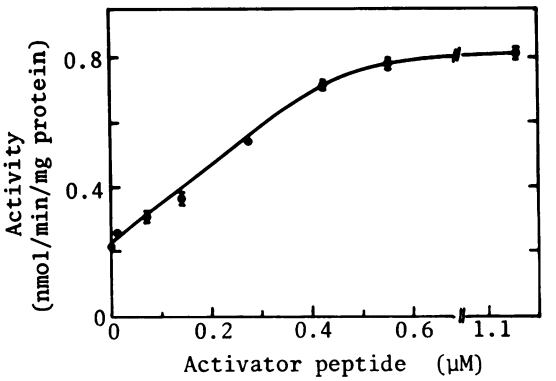

FIG. 6. Effect of activator peptide concentration on cholesterol scc activity. Values reflect mean ± SD of triplicate incubations. Absence of error bars indicates an error less than symbol height.

The effect of the activator peptide on cholesterol scc activity is rapidly expressed *in vitro* (Fig. 5). This apparently results from an increased association of cholesterol with the cytochrome P-450$_{scc}$ complex, as indicated by the increased pregnenolone-induced type II absorbance change after addition of activator peptide to mitochondria prepared from the adrenals of hypophysectomized rats. Because a linear increase in cholesterol scc activity is dependent on linear (rather than exponential) increases in activator peptide concentration (Fig. 6), enzyme activation is probably not mediated by an amplifying cascade of intermediate reactions. Further kinetic studies are warranted.

The highest level of cholesterol scc activity achieved with the activator peptide in this study (4- to 5-fold increase over basal) is about half the increment observed in adrenal mitochondria isolated from animals fully stimulated with ACTH (36). It may be possible to narrow this margin by further optimizing the reconstitution conditions. Moreover, Neher *et al.* (18) have demonstrated a synergistic activation of mitochondrial cholesterol conversion by unidentified factors when a combination of subcellular fractions was used. Also, recent studies by Farese *et al.* (37, 38) suggest a role for phospholipids in the adrenal steroidogenic response to ACTH. Some functional relationship between the activator peptide and membrane phospholipid is therefore conceivable.

The charge on the peptide is uncertain because of inherent Asx and Glx ambiguity. However, the prolonged retention of the intact peptide during reverse-phase HPLC (Fig. 4) is predicted (39) if one assumes either a predominance of Asp and Gln or some unidentified structural feature(s). The retention period is also consistent with peptide hydrophobicity. The cysteine content is noteworthy because it suggests the potential for an intrachain disulfide bridge. Moreover, because sample extracts were not reduced before purification, peak I activity (Sephadex G-25 chromatography; Fig. 2) may represent polymerization of the material in peak II. It is noteworthy that peak I activity was lost when whole adrenals were homogenized directly in acidic medium (Table 1), avoiding the increased potential for disulfide interchange during subcellular fractionation at neutral pH.

Attempts at micro sequence analysis of the peptide by au-

tomated Edman degradation using conventional and gas-phase (40) instruments were unsuccessful, and no product was detected after incubation of the peptide with aminopeptidase M. This suggests that the amino terminus of the peptide is blocked. Consequently, other strategies will be necessary to derive the structure.

The peptide isolated in this study can be distinguished from other adrenal activating factors that have been described. First, this peptide is smaller than the sterol carrier proteins. Nevertheless, its effect on adrenal cholesterol scc is similar to that reported for a sterol carrier protein from bovine liver (20), so some functional relationship may exist between the two. Unlike the adrenal activator described by Kan and Ungar (16), this peptide is sensitive to ACTH. Its smaller size and heat stability also distinguish it from the cytosolic activators reported by Farese (15) and by Ray and Strott (17). Dazord *et al.* (41) have demonstrated an ACTH-stimulated increase in a 3,500-dalton peptide isolated from mouse adrenal tumor cells, but the temporal response suggests protein induction rather than activation. Lieberman and colleagues (19) have reported the isolation of a cholesterol scc activator from bovine adrenal, but the relationship between that material and the peptide described here is still uncertain.

We speculate that this novel adrenal peptide may be translated *de novo* in response to ACTH. Alternatively, it could arise from a larger, constitutive protein susceptible to post-translational proteolytic processing. One can envision this limited proteolysis under ACTH control, perhaps via cAMP-mediated enzyme phosphorylation. Generation of a labile activator peptide from inactive precursor in this way would afford a sensitive mechanism for hormonal modulation of adrenal cholesterol scc. Further structural characterization of the peptide and the development of a sensitive means for its detection, such as a radioimmunoassay, should permit the investigation of these and related hypotheses.

The MtT/F4 pituitary tumor was a gift of the Breast Cancer Task Force Tumor Bank, National Cancer Institute. We thank Drs. N. Whiteley (Applied Biosystems, Foster City, CA) and M. Garrick for micro sequence analysis attempts with gas-phase and conventional peptide sequenators, respectively. The expertise of Mr. L. Joseph and Dr. J. Alfano with the animal experimentation is gratefully acknowledged. Competent technical assistance was also provided by Ms. S. Siembida, Ms. M. P. Russo, and Mr. R. Linsmair. Dr. S. Gallant provided helpful advice throughout. This study was supported by Public Health Service Grants AM-18141 and HL-06975.

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