Regulation of cholesterol ester hydrolase by cyclic AMP-dependent protein kinase

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Received 18 April 1986

Phosphorylation of cholesterol ester hydrolase by cyclic AMP-dependent protein kinase results in activation of both cholesterol ester and triacylglycerol hydrolase activities. Activation against both substrates correlates closely with phosphorylation in time course experiments. Proteolytic digestion of phosphorylated cholesterol ester hydrolase, followed by peptide mapping, indicates the presence of a single phosphorylation site on the enzyme. Phosphoserine is the only phosphoamino acid detected following partial acid hydrolysis of the phosphorylated enzyme.

Cholesterol ester hydrolase *Phosphorylation* *Steroidogenesis*

1. INTRODUCTION

Cholesterol for steroidogenesis can be derived from three distinct sources, i.e. by uptake from lipoproteins, by de novo synthesis or from intracellular cholesterol ester stores. Supply of cholesterol to the mitochondrial cytochrome $P450_{scc}$ for conversion to pregnenolone is generally considered to be the rate-limiting step in steroidogenesis [1]. Cholesterol ester hydrolase is the enzyme responsible for the release of cholesterol from the cholesterol ester stores and, as such, is a potential point for regulation of steroidogenesis.

Much evidence has accumulated supporting the concept that cholesterol ester hydrolase is regulated by reversible phosphorylation by cyclic AMP-dependent protein kinase. Administration of adrenocorticotropic hormone (ACTH) or luteinising hormone (LH), which are thought to act via cyclic AMP, elevates the activity of cholesterol ester hydrolase in extracts of adrenal cortex [2,3] and ovary [4,5], respectively. The activity of partially purified preparations of the enzyme is also elevated by incubation with cyclic AMP-dependent protein kinase and ATP-Mg^{2+} [6,7]. Inclusion of the inhibitor protein of cyclic AMP-dependent protein kinase blocks this activation [6], indicating that cholesterol ester hydrolase serves directly as a substrate for the kinase.

The cholesterol ester hydrolase protein has been identified as having an M_r of 84000, and has been partially purified from bovine adrenal cortex [8] and corpus luteum [9]. Interestingly the enzyme shares many properties with purified hormone-sensitive lipase from rat adipose tissue [10], an enzyme also regulated by phosphorylation by cyclic AMP-dependent protein kinase. This has led to the suggestion that the 84 kDa protein serves as a hormone-sensitive triacylglycerol and cholesterol ester hydrolase in several tissues, with the function of the enzyme being dictated by the substrate available to it in any particular tissue. Partially-purified cholesterol ester hydrolase from both adrenal cortex and corpus luteum has been shown to become phosphorylated by cyclic AMP-dependent protein kinase. However phosphorylation of this partially purified enzyme has to date only been shown to be associated with activation against a trioleoylglycerol substrate [9,10] and no

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

details of the phosphorylation of the enzyme have been reported.

Here we present evidence that phosphorylation by cyclic AMP-dependent protein kinase activates the enzyme against both cholesterol oleate and trioleoylglycerol substrates. Evidence is also presented that the phosphorylation catalysed by this kinase occurs at a single serine residue on cholesterol ester hydrolase.

2. MATERIALS AND METHODS

V8 protease was from Miles Laboratories, trypsin from Worthington and hexokinase from Boehringer. [γ-^{32}P]ATP was synthesised according to Glynn and Chappell [11], and isolated by the method of England [12]. All other chemicals and biochemicals were of the highest available purity, from either BDH or Sigma.

Cholesterol ester hydrolase was routinely assayed against cholesterol oleate using an ethanolic suspension assay [8]. Following phosphorylation, the enzyme was assayed for activation either at pH 7.0 against cholesterol oleate in phosphatidylcholine/taurocholate micelles [13] or at pH 8.3 against trioleoylglycerol in phosphatidylcholine/phosphatidylinositol micelles [14] as indicated.

Cholesterol ester hydrolase was partially purified from bovine adrenal cortex or corpora lutea as described [8,9]. The enzyme was eluted from hydroxyapatite with a gradient (5–200 mM) of sodium phosphate and then concentrated to approx. 10 mU/ml (as determined by the ethanolic suspension assay; see above). Alternatively, the enzyme was purified by a new procedure involving isoelectric precipitation followed by successive chromatographies on DEAE-cellulose, phenyl-Sepharose and heparin-Sepharose [15].

The catalytic subunit of cyclic AMP-dependent protein kinase was purified from the supernatant of the pH 5.2 precipitation step of a cholesterol ester hydrolase preparation from adrenal cortex according to Strålfors and Belfrage [16]. The procedure was modified so that the first CM-Sephadex column was washed with 50 mM potassium phosphate, pH 6.6, and then eluted with a gradient of 50–350 mM potassium phosphate at the same pH. No further purification was attempted and the pooled fractions were concentrated to approx. 150 U/ml (assayed as in [17]). Phosphorylation of cholesterol ester hydrolase was performed routinely by incubation in the presence of 0.2 mM ATP, 2 mM $MgCl_2$ and the catalytic subunit of cyclic AMP-dependent protein kinase. For activation determinations phosphorylation was performed using non-radioactive ATP and the reaction stopped by the addition of glucose and hexokinase to final concentrations of 0.1 M and 10 U/ml, respectively. Phosphorylation was determined following incubation with [γ-^{32}P]ATP. In this case the reaction was terminated by the addition of trichloroacetic acid and acetone to 10% (w/v) and 30% (v/v), respectively. The precipitated protein was analysed by SDS-polyacrylamide gel electrophoresis and autoradiography. The extent of phosphorylation of cholesterol ester hydrolase was determined by scanning densitometry of the autoradiographs followed by integration. The area under the 84 kDa phosphorylated protein was taken as a measure of phosphorylation.

Phosphorylated cholesterol ester hydrolase was extracted from SDS-polyacrylamide gel slices as described in [18]. After precipitation with 20% (w/v) trichloroacetic acid using 0.01 mg DNA as carrier, the protein was subjected to partial acid hydrolysis under vacuum in 6 N HCl at 95°C for 3 h. Alternatively, phosphorylated protein was subjected to proteolytic digestion using 0.5 mg/ml V8 protease in 0.1 ml of 0.5% ammonium bicarbonate. After 5 h at 37°C, digestion was terminated by heating to 100°C for 1 min. Trypsin and $CaCl_2$ were then added to 0.1 mg/ml and 2 mM, respectively, and digestion allowed to continue overnight.

3. RESULTS AND DISCUSSION

As reported [10] phosphorylation of the 84 kDa polypeptide of cholesterol ester hydrolase by cyclic AMP-dependent protein kinase activates the enzyme against a trioleoylglycerol substrate. Furthermore the data in fig.1 also demonstrate that phosphorylation of the enzyme activates the enzyme when assayed against cholesterol esters at the physiological pH of 7.0. In a series of 32 experiments using cholesterol oleate as substrate and using enzyme from both adrenal cortex and corpus luteum a mean activation of 58% (SE 4%, range 20–91%) was observed over control incubations

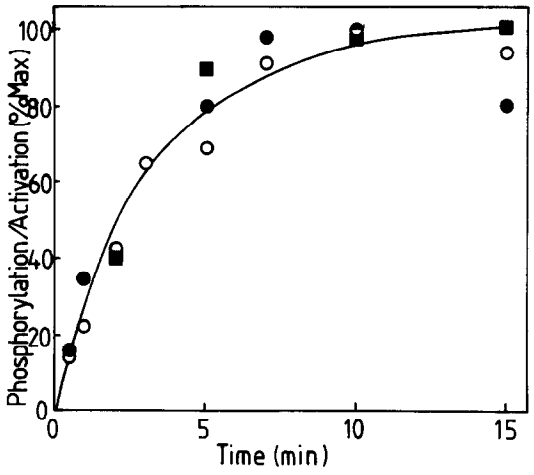

Fig.1. Activation and phosphorylation of cholesterol ester hydrolase. Enzyme from corpus luteum was incubated at 37°C with 0.2 mM ATP (●, ■) or 0.2 mM [γ-^{32}P]ATP (○), 2 mM $MgCl_2$ and 1.5 units/ml of the catalytic subunit of cyclic AMP-dependent protein kinase. At the indicated times aliquots were removed and phosphorylation terminated as described in section 2. The non-radioactive incubation was then assayed for activation against triacylglycerol (■) and cholesterol oleate (●) substrates whilst the radioactive incubation was assayed for phosphorylation (○). All values are expressed as a percentage of the maximum value observed.

carried out in the absence of ATP-Mg^{2+}. The large range in observed activation is thought to be due to variation in the substrate presentation in different experiments, rather than different stoichiometries of phosphorylation. No significant difference was observed in the behaviour of the enzyme from the two sources.

In time course experiments phosphorylation of the 84 kDa enzyme subunit appeared complete within 10 min (fig.1). Accurate determination of the stoichiometry of phosphorylation was not possible because of the low degree of purity of the enzyme but based on an assumed specific activity of 200 units/mg for homogeneous enzyme [14] a stoichiometry of phosphorylation of approx. 0.4 mol phosphate/mol subunit is estimated. In parallel experiments with non-radioactive ATP, activation of both the triacylglycerol and cholesterol ester hydrolase activities of the enzyme followed the same time course and correlated closely with phosphorylation of the protein (fig.1), providing further evidence that both activities are catalysed by the same enzyme protein [8]. In addition, it also suggests that cyclic AMP-dependent protein kinase phosphorylates a single site on the enzyme and that this phosphorylation is directly responsible for the observed activation.

Partial acid hydrolysis of isolated phosphorylated cholesterol ester hydrolase reveals that serine is the only amino acid residue in the protein which is phosphorylated by cyclic AMP-dependent protein kinase (fig.2). Phosphorylated cholesterol

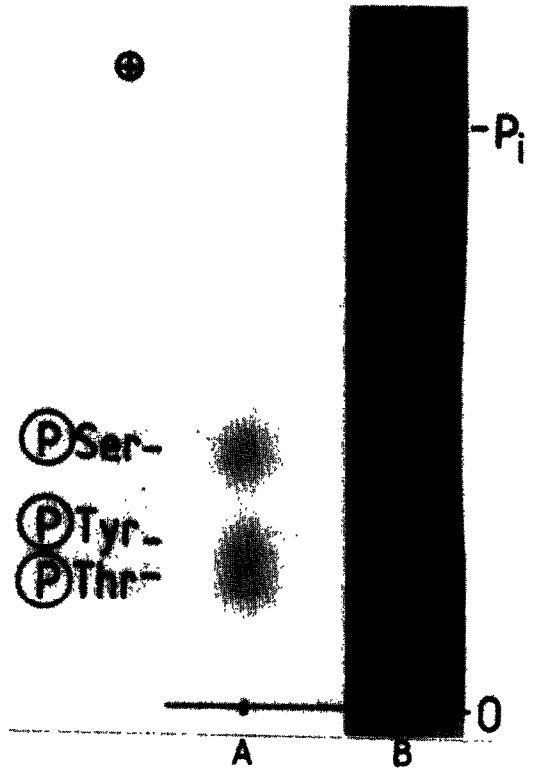

Fig.2. Phosphoamino acid analysis of cholesterol ester hydrolase. Enzyme from corpus luteum was phosphorylated by incubation with 10 units/ml catalytic subunit of cyclic AMP-dependent protein kinase, 5 mM $MgCl_2$ and 0.15 mM [γ-^{32}P]ATP for 30 min at 37°C. The phosphorylated enzyme protein was then isolated and subjected to partial acid hydrolysis as described in section 2. After drying over solid NaOH, the hydrolysate was analysed by high-voltage electrophoresis at pH 1.9 (2 kV, 30 min) with 20 nmol of each standard phosphoamino acid. Lane A shows a ninhydrin stain of the electrophoretogram and lane B the corresponding autoradiograph.

ester hydrolase was also subjected to proteolytic digestion, using V8 protease and trypsin [19]. Analysis of the digest by high-voltage electrophoresis on paper at pH 1.9 indicated the presence of a single phosphopeptide (fig.3), with mobility identical to that of the corresponding phosphopeptide from bovine adipose tissue hormone-sensitive lipase. Similarly electrophoresis on silica at pH 3.4

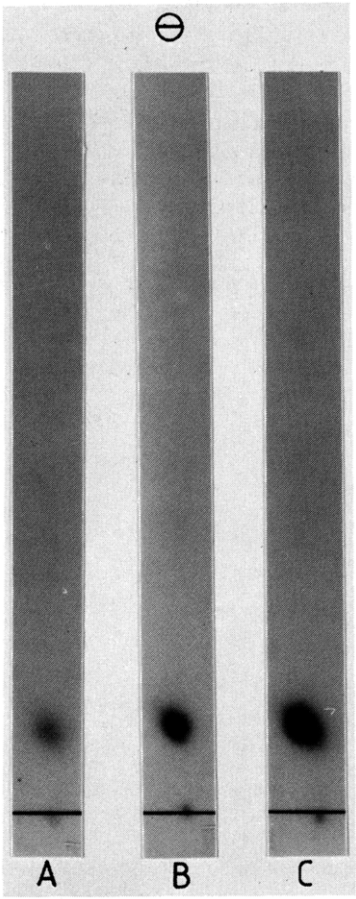

Fig.3. High-voltage electrophoresis of phosphopeptide from cholesterol ester hydrolase from corpus luteum. The enzyme was phosphorylated and isolated as in fig.2. After washing the protein pellet with acetone and then diethyl ether, the protein was digested as in section 2. After freeze-drying, the crude digest was re-dissolved in 7% formic acid and subjected to paper high-voltage electrophoresis at pH 1.9 at 2.5 kV for 45 min. An autoradiograph of the electrophoretogram is shown. (A) Corpus luteal cholesterol ester hydrolase, (B) bovine adipose tissue hormone-sensitive lipase, (C) mixture of the two enzymes.

revealed the presence of a single acidic phosphopeptide (not shown) again with mobility identical to that of the corresponding phosphopeptide from adipose tissue hormone-sensitive lipase [15,19]. Ascending chromatography on silica again indicated that the phosphopeptides from the different sources have very similar or identical properties, although the phosphopeptide from cholesterol ester hydrolase frequently splits into two spots on chromatography, perhaps due to partial oxidation (not shown). The phosphopeptide from cholesterol ester hydrolase of adrenal cortex and corpus luteum contains about 8 amino acids as determined by gel-filtration chromatography on a calibrated column of Sephadex G25 (not shown). In view of the small size of this peptide it is extremely unlikely that it contains more than one phosphorylated residue and all the data are consistent with the presence of a single phosphorylation site. Furthermore the behaviour of the phosphopeptide from the enzyme from steroidogenic tissues is essentially identical to that of the corresponding peptide from adipose tissue hormone-sensitive lipase, providing further evidence for the possible identity of these two enzymes [10].

These results extend our knowledge of the regulation of cholesterol ester hydrolase by covalent phosphorylation and provide the first direct evidence that phosphorylation of the enzyme correlates with increased activity against its physiological substrate. The similarity between the data reported here concerning the phosphorylation site on the steroidogenic cholesterol ester hydrolase and that reported [15,19] for regulation by cyclic AMP-dependent protein kinase of the adipose tissue hormone-sensitive lipase further substantiates the relationship between the two enzymes [10]. Furthermore it will now be possible to investigate at the molecular level the possible phosphorylation of cholesterol ester hydrolase in steroidogenic tissues in response to the appropriate tropic hormones.

ACKNOWLEDGEMENTS

This work was supported by grants from the Medical Research Council, UK and the British Diabetic Association. R.J.C. held a studentship from the Medical Research Council. A.J.G. holds a studentship from the Science and Engineering

Research Council, UK. S.J.Y. is a Lister Institute Research Fellow.

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