Regulation of Steroid Hormone Biosynthesis
IDENTIFICATION OF PRECURSORS OF A PHOSPHOPROTEIN TARGETED TO THE MITOCHONDRIAN STIMULATED RAT ADRENAL CORTEX CELLS

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Two short-lived precursor proteins, pp37 and pp32, of the mitochondrial phosphoprotein pp30 (formerly denoted as iβ) have been detected in β,β-cAMP-stimulated rat adrenal cortex cells, incubated at 25 °C or with 1,10-orthophenanthroline at 37 °C. Subsequently, these two precursor proteins were also identified in cells incubated at 37 °C, where they are present only at low levels due to their short half-life. pp30 is produced in several steroidogenic tissues in response to trophic hormone or second messenger analogue. pp37 and pp32 are also phosphoproteins located in the mitochondrion that are produced in response to cAMP analogue and give rise to proteolytic peptide maps similar to that of pp30. As for pp30, inhibition of cytosolic translation prevents the production of pp37 and pp32. The larger precursor protein pp37 has an apparent molecular mass of 37 kDa, an isoelectric point of ~7.1, and a half-life at 37 °C of ~3-4 min. Pulse-chase studies indicate that this protein is processed into the smaller protein, pp32, which has an apparent molecular mass of 32 kDa, an isoelectric point of ~6.4, and a half-life at 37 °C of 3-4 min. This latter protein is the immediate precursor of pp30. Since orthophenanthroline inhibits the mitochondrial processing protease, while the lower incubation temperature slows both protein import and protease processing, the experimental conditions necessary to detect these proteins are consistent with pp37 being a precursor protein that contains two cleavable presequences and is imported into the mitochondrion. The sequential removal of these sequences produces the mature protein pp30.

The acute action of ACTH on adrenal cortex cells is receptor-mediated (1-2) and results, after a lag of approximately 3 min, in a 10-15-fold increase in the rate of steroid hormone synthesis (3-5). This enhanced rate of steroidogenesis is produced by an increased availability (6) of the substrate cholesterol to cytochrome P-450α, located in the inner mitochondrial membrane (7-8) and results in an increase in the rate of pregnenolone synthesis (9-10). This mitochondrial cytochrome with its auxiliary electron transferring proteins catalyzes the initial (11, 12) and rate-determining reaction (10) in the corticosteroid synthetic pathway. Previous studies have shown that cAMP levels increase upon exposure of the cells to ACTH (13, 14) and that cAMP-dependent events are required for the acute stimulation (15-17), most importantly, the activation of cAMP-dependent protein kinase (18-20). Furthermore, stimulation of steroidogenesis has been shown to require continuous protein synthesis (21, 22). Addition of a translation inhibitor, such as cycloheximide (CHx), prior to addition of peptide hormone or second messenger analogue, prevents the increase in the rate of steroidogenesis. Further, the addition of the inhibitor, subsequent to the addition of stimulant, causes steroidogenesis to return within ~5 min to its low, basal velocity. These data are most easily interpreted if the subcellular events that cause increased steroidogenesis depend on the synthesis of a "labile protein" (23-24), i.e. a protein that must be synthesized continuously because it is degraded continuously. It has been postulated that the function of this protein is to facilitate the access of cholesterol to cytochrome P-450α in the mitochondrion (25). Subsequently, it was demonstrated directly that the accumulation of cholesterol in the inner mitochondrial membrane of stimulated cells is prevented by inhibition of cytosolic translation (6).

We have detected previously, in several steroidogenic tissues, a mitochondrial phosphoprotein pp30 (formerly called iβ), whose appearance correlates with the increase in the rate of steroidogenesis following stimulation (26-31). Since inhibition of cytosolic protein synthesis inhibits production of pp30, either pp30 or a longer precursor of this protein is encoded in the nucleus, synthesized on cytoplasmic ribosomes, and imported into the mitochondrion. p30, the unphosphorylated homologue of pp30, accumulates in unstimulated cells and is also localized in the mitochondrion. However, pulse-chase studies have demonstrated that p30 is not converted into pp30 by post-translational phosphorylation. Therefore p30 is not an intermediate in the synthesis of pp30. Upon addition of CHx, production of pp30 ceases and the protein is converted post-translationally into a structurally similar, more acidic protein pp30'. However, this conversion does not proceed as rapidly as the cessation of stimulated steroidogenesis when a translation inhibitor is added. Therefore, it was assumed that the lability reflected inactivation of function rather than actual degradation of the protein. Since pp30 is localized to the mitochondrion and since its production depends on cytosolic translation, we decided to slow or inhibit the translocation of proteins into the mitochondrion in an attempt to detect possible precursors of pp30 and to measure the rates of their import and proteolytic processing.

In general, nuclear-encoded proteins that are targeted to the mitochondrion are translated as longer precursors (32-
were stored at -20 °C until use. pp37 carries cholesterol with it during this transit process. Labeling mix ([35S]Met/Cys), containing 77% methionine and 18% alkaline phosphatase were obtained from Sigma. 1,1O-Orthophenanthroline monohydrate was purchased from Aldrich Chemical Co. New England Nuclear. Ampholyte were purchased from Pharmacia culture reagents were from Bio-Rad or Sigma. X-omat XAR-5 film was from Kodak. Other chemicals were of the highest purity available.

In the study presented here, we describe the results of experiments in which techniques, common to the study of mitochondrial protein, were used to detect these two longer precursors of pp30. The precursors are processed by proteolytic cleavage into mature pp30 so readily that it is difficult to detect them at 37 °C. Since pp30 precursor must be translocated across both mitochondrial membranes, it is possible that such precursor proteins are imported into the mitochondrion (40-42). Such targeting sequences lack acidic amino acid residues and are rich in hydroxylated and in positively charged amino acids, and thus are capable of forming amphipathic helices (43, 44). In addition to this sequence, some proteins contain, adjacent to the organelle-targeting sequence, a cleavable sequence that directs the protein either to the inner membrane (e.g. cytochrome c,) or into the intermembrane space (e.g. cytochrome b) (45-47).

This latter sequence is removed by a protease located in the inner membrane (48). Thus, for proteins such as cytchrome bs and c1, the protein that is translated in the cytosol is processed by two sequential proteolytic steps into the mature mitochondrial protein. Another type of sequential proteolytic cleavage has been documented for the processing of the matrix enzymes malate dehydrogenase and ornithine transcarbamoylase in rat liver (38, 39). This sequential cleavage is accomplished by two distinct processing proteases located in the matrix.

At this point it is not possible to state definitively which of the two processing pathways described above accomplishes the production of the mature protein pp30. However, the fact that pp30 is an integral membrane protein might be more consistent with the second cleavage event being the removal of a sorting sequence by the inner membrane protease (48). In the study presented here, we describe the results of experiments in which techniques, common to the study of mitochondrial protein import, were used to detect these two longer precursors of pp30. The precursors are processed by proteolytic cleavage into mature pp30 so rapidly that it is difficult to detect them at 37 °C. Since pp30 precursor must be translocated across both mitochondrial membranes, it is possible that pp37 carries cholesterol with it during this transit process.

**EXPERIMENTAL PROCEDURES**

**Materials**

Female Sprague-Dawley rats, weighing 151-200 g, were purchased from Taconic Farms (Germantown, NY) and used after 1-2 days. ACTH, cycloheximide, Bt2-cAMP, chymotrypsin, collagenase Type 1, desoxyribonuclease 1, bovine serum albumin, and Escherichia coli alkaline phosphatase were obtained from Sigma. 1,10-Orthophenanthroline monohydrate was purchased from Aldrich Chemical Co. Ham's F-12 and calf serum were purchased from GIBCO. [35S]Met/Cys, containing 77% methionine and 18% cysteine, and [32P]orthophosphate were purchased from Du Pont-New England Nuclear. Amphoties were purchased from Pharmacia LKB Biotechnology Inc. All other electrophoresis reagents and cell culture reagents were from Bio-Rad or Sigma. X-omat XAR-5 film was from Kodak. Other chemicals were of the highest purity available commercially.

**Methods**

**Cell Isolation and Subcellular Fractionation Procedures**—The collagenase digestion procedure for rat adrenals was adapted from that of Ray and Strott (49) as described previously (26). Adrenal cells (2.0 \times 10^6 cells/ml) were osmotically lysed and fractionated by centrifugal pelletization essentially according to the method of DiBartolomeis and deRota (50) as described previously (51). The final cell and mitochondrial pellets and post-mitochondrial supernatant fraction were stored at -20 °C until use.

Incorporation of Radiolabeled Amino Acids into Proteins—All cell incubations were carried out in Kreb's ringer bicarbonate buffer (KRBA) (51), containing 0.5% bovine serum albumin (w/v) and 25 mM HEPES, pH 7.4, at 37 °C, with gentle agitation. For radiolabeling, cells were incubated with 1 mc/ml of [35S]Met/Cys at a cell concentration of 0.5 or 1 \times 10^6 cells/ml. Aliquots for two-dimensional polyacrylamide gel electrophoresis (see below) were quenched with a 3-fold volume of KRBA at 4 °C and centrifuged at 100 X g for 12 min to pellet cells. The supernatants were discarded and pellets stored at -20 °C until use. The cells were solubilized in Laemmli SDS sample buffer (52) as described previously (27). [35S]Met/Cys incorporation into total cellular protein was assayed in competition with trichloroacetic acid and scintillation counting by a modification described previously (27) of the method of Ilan and Ilan (53).

Incorporation of [32P]Orthophosphate—Rat adrenal cells were isolated and purified as described above. The cells (4 \times 10^6) were washed twice with sterile Ham's F-12 containing 10% calf serum, 1% penicillin-streptomycin, resuspended in 12 ml of this medium, distributed among six 35-mm plastic Petri dishes, and incubated for 18 h at 37 °C, under a humidified atmosphere of 5% CO2. The anchored cells were washed three times with phosphate-free Earle's balanced salt solution (EBSS) immediately prior to the addition of radiolabel. [32P]Orthophosphate (0.5 mc/ml) in EBSS was added to the cells together with Bt2-cAMP or with Bt-cAMP and CHX and incubation continued as before, for 2 h with frequent agitation. At the end of the incubation, the cells were washed four times with phosphate-buffered saline (PBS), pH 7.4, at 4 °C, scraped off the dish in 0.5 mls of PBS, pooled, and solubilized in Goerres' sample buffer for two-dimensional gel electrophoresis (54, 55).

**Electrophoresis Methodology**—Isoelectric focusing, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), two-dimensional polyacrylamide gel electrophoresis (2-D PAGE), alkaline phosphatase treatment, proteolytic peptide mapping, autoradiography ([35S]-labeled samples), and fluorescence ([32P]-labeled samples), were all performed by modifications of published procedures (52-56) as described previously (27-28). A Cronex lightning-plus intensifying screen (Du Pont) was used for exposure of x-ray film to [32P]-labeled gels.

Quantitation of Radiolabeled Amino Acid Incorporation into Specific Proteins—Quantitation of incorporation of radiolabeled amino acid into specific proteins of interest was carried out using a BioImage Visage 110 (BioImage, Ann Arbor, MI) computer-assisted image analysis system. Unless otherwise noted in the figure legends, equal amounts of trichloroacetic acid-precipitable protein counts were loaded onto the gels in any group of experiments, and the film was exposed for the same length of time. Further, the amount of radioactive protein actually entering the gels was calculated by comparison of the integrated intensities of "standard" spots for all gels within a given experiment. These standard spots were proteins that migrated in the same region of the gel as the protein of interest. The intensity of these spots was summed. This sum was taken as a measure of the amount of radioactive protein entering the gel and used to correct the intensities of the spots of interest. Using this method, the amount of protein entering the gel was found to differ by at most 20% between the samples for such experiment (data not shown). Additionally, for pulse-chase experiments, the intensity was taken to be 100% for the maximum amount of precursor or final product. Thus, the amount of pp37 present at t = 0 was taken to be 100%. The usage of the 100% formalism allowed us to pool data from samples with different times (therefore amount of radiolabel incorporation (see below).

**RESULTS**

**Occurrence in cAMP-stimulated Cells of Higher Molecular Weight Proteins with Primary Structure Similar to pp30 (a),**—The acute stimulation of corticosteroidogenesis by ACTH is mimicked by an analog of cAMP, Bt-cAMP, of the second messenger type, including Bt2-cAMP and 8-bromo-cAMP. Thus, we have used Bt-cAMP to produce stimulation in these studies. Fig. 1 shows fluorograms of two-dimensional gels of rat adrenal cortex cell preparations incubated at 25 °C for 15 min with [35S]Met/Cys in the absence or presence of Bt2-cAMP and of cells exposed to radiolabeled amino acid for 15 min prior to the addition of this cAMP analogue plus the translation inhibitor CHX. At this temperature, Bt-cAMP causes the increased accumulation of two proteins detected previously only in very low
FIG. 1. Differences in the levels of specific radiolabeled proteins in adrenal cells incubated at 25 °C. Rat adrenal cortex cells (1 X 10^6 cells/ml) were incubated for 15 min at 25 °C with 1 mCi/ml [35S]Met/Cys in the absence (control) or presence (cAMP) of 2 mM Bt2cAMP. Samples that were treated with CHx (cAMP + CHx) were exposed to radiolabeled amino acids for 15 min at 25 °C. Then, 0.2 mM CHx and 2 mM Bt2cAMP were added and the incubation continued for an additional 15 min. Equal trichloroacetic acid-precipitable counts were loaded onto two-dimensional gels, and the fluorograms were produced by exposure of x-ray film for the same length of time as described under "Experimental Procedures." The position of migration of standard molecular mass marker proteins is shown in kilodaltons at the left and the measured, approximate values for the pH gradient are shown below the gels. These gels are representative of the results of eight such experiments.

One protein has an apparent molecular mass of 37 kDa and the other, an apparent molecular mass of 32 kDa. Additionally, in studies at higher temperatures, pp30 and its post-translational product pp30' accumulate upon addition of Bt2cAMP (26-28). These four proteins are indicated by arrows and labels in the center panel of the figure. In the gel from control cells, we have also identified p30 and its post-translational product p30', the unphosphorylated homologues of pp30, and pp30', respectively. As is also apparent from Fig. 1, inhibition of protein synthesis by CHx at the time of stimulation prevents the appearance of the two new proteins as well as the appearance of pp30 and pp30'. Small downward pointing arrows on the bottom panel indicate the positions where these four proteins would be seen; the proteins p30 and p30' are indicated in this panel. p30 is synthesized during the initial labeling period prior to the addition of Bt2cAMP and CHx. Thus, these data confirm our previous observation that p30 is not converted into pp30 post-translationally (26, 27). As shown by the proteolytic peptide mapping patterns in Fig. 2, the two new proteins that can be detected using this lower incubation temperature are similar in primary structure to pp30 and pp30'. Although each has higher molecular weight fragments, the lower molecular weight fragments from all four proteins are similar. For comparison, the proteolytic peptide mapping patterns of other unrelated proteins in this region of the gel are also shown.

**Demonstration That the 37- and the 32-kDa Proteins Are Phosphorylated**—Fig. 3 shows autoradiograms of two-dimensional gels to allow comparison of the protein phosphorylation patterns of stimulated (Bt2cAMP) and of unstimulated (control) cells, both exposed to [32P]orthophosphate. Additionally, an autoradiogram of cells exposed to both Bt2cAMP and CHx is shown. The positions of migration of the 37- and 32-kDa proteins and of pp30 and pp30' are indicated in the center panel of Fig. 3. Since these proteins are shown to be phosphoproteins, the higher molecular weight proteins are denoted

**FIG. 2. Similarity of chymotryptic peptides of the 37- and the 32-kDa rat adrenal cortex proteins to pp30 and pp30'.** Gel pieces containing the proteins of interest were excised, placed into the well of a stacking gel containing 1 µg of chymotrypsin, and the proteolytic peptide fragments were separated by SDS-PAGE on e 15% gel, as described by Cleveland (56). The figure is a composite of two different exposures of the same gel. The lane numbers on the top of the one-dimensional gel correspond to the numbered spots on the bottom two-dimensional gel. Lane 1 is pp30; lane 2, pp30'; lane 3, 37-kDa protein; lane 4, 32-kDa protein; lane 5, unrelated 30-kDa protein; and lane 6, unrelated 38-kDa protein.
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pp37 and pp32. Thus, phosphoproteins with the correct electrophoretic mobilities for all four proteins are detected in the cells exposed to Bt2cAMP. These four proteins, whose positions of migration are indicated by the small downward pointing arrows in the top and bottom panels, are not detected in control or CHx-treated samples. This indicates that the phosphorylation occurs only in stimulated cells and on the newly synthesized protein. This is in contrast to two other phosphoproteins labeled 1 and 2 in the cAMP-stimulated sample that are also present in the cAMP + CHx-treated sample. These latter proteins are phosphorylated post-translationally in response to cAMP, since their phosphorylation is not influenced by the inhibition of translation. Confirmation of the mobility of the proteins of interest in samples treated with this protocol was obtained by labeling plated cells with \([35S]\)Met/Cys under conditions identical to those used for the \([32P]\)orthophosphate incorporation study (data not shown).

Fig. 4 shows fluorograms of two-dimensional gels of stimulated cells labelled at 25 °C with \([35S]\)Met/Cys and subsequently solubilized and incubated with and without \(E.\ coli\) alkaline phosphatase \textit{in vitro} (28). The change in mobility, subsequent to alkaline phosphatase treatment, of the 37- and the 32-kDa proteins, as well as pp30 and pp30', which has been documented previously (28), confirms that all four proteins are phosphorylated. The reaction was not taken to completion. Therefore, some of the phosphorylated forms remain. Quantitation of the gels indicates that 80-90% of pp37 and of pp30 has served as a substrate for the phosphatase. The dephosphorylated form of pp37 is not detected, presumably because its PI is too basic for the protein to be focused on the gel, and thus no recovery may be calculated. However, the recovery of p30 from pp30 is essentially quantitative. The two newly detected proteins are thus found to be phosphoproteins as determined both directly by phosphate incorporation and indirectly by monitoring the change in electrophoretic mobility produced by incubation of the proteins with phosphatase. Therefore, the proteins will be designated pp37 and pp32.

![Fluorograms of two-dimensional gels of stimulated cells labelled at 25 °C with \([35S]\)Met/Cys and subsequently solubilized and incubated with and without \(E.\ coli\) alkaline phosphatase.](image)

*Fig. 3. Phosphorylation of proteins monitored by \([32P]\)-orthophosphate incorporation in control and Bt2cAMP-stimulated adrenal cells.* Rat adrenal cortex cells were isolated and placed on tissue culture plates (3.5 × 10⁶ cells/35-mm² plate) as described under “Experimental Procedures.” Bt2cAMP or Bt2cAMP and CHx were added concurrently with the addition of \([32P]\)orthophosphate. The concentration of Bt2cAMP was 2 mM, of CHx, 0.2 mM, and of \(^{32}\)P-label, 0.5 mCi/ml. After the incubation, the entire sample was solubilized and the proteins were separated by 2-D PAGE as described under “Experimental Procedures.” The proteins of interest are labeled as in Fig. 1; additionally, two proteins that are phosphorylated in cAMP-dependent, post-translational events are indicated by the numbers 1 and 2. This experiment was carried out three separate times with identical results.

![Fluorograms of two-dimensional gels of stimulated cells labelled at 25 °C with \([35S]\)Met/Cys and subsequently solubilized and incubated with and without \(E.\ coli\) alkaline phosphatase.](image)

*Fig. 4. Confirmation of identification of phosphoproteins by alkaline (alk.) phosphatase (phos.) treatment of proteins visualized by radiolabeled amino acid incorporation.* Rat adrenal cortex cells were isolated and incubated as for Fig. 1. The cell pellet was solubilized and aliquots containing equal trichloroacetic acid-precipitable counts were incubated for 30 min at room temperature with or without \(E.\ coli\) alkaline phosphatase, as described previously (28). The phosphatase reaction was terminated by the addition of Garrels' sample buffer, and the proteins from each sample were separated by 2-D PAGE. The fluorograms are labeled as for Fig. 1. The large arrowheads in the upper panel (no alkaline phosphatase) show the positions of migration of pp37, pp32, pp30, and pp30'. The large arrowheads in the lower panel (plus alkaline phosphatase) show the positions of migration of p30, p30', and p32 (large unlabeled arrowhead). Identical data were obtained from two independent experiments.
Mitochondrial Localization of pp37 and pp32—Fluorograms of two-dimensional gels of subcellular fractions of [³⁵S]Met/Cys-labeled rat adrenal cortex cells exposed to Bt₂cAMP are shown in Fig. 5. As in the experiments described above, the cells were labeled for a short time at 25 °C to produce a higher concentration of the two higher molecular weight phosphoproteins. As has been shown previously for p30 and pp30, and their post-translational products, p30' and pp30', respectively, pp37 and pp32 are localized to the mitochondrion and are not found in the cytosol. This result is consistent with these proteins being precursors of pp30 that are converted into this protein by proteolysis within the mitochondrion (see below).

Effect of 1,10-Orthophenanthroline on the Accumulation of pp37—Fig. 6 shows fluorograms of two-dimensional gels which illustrate the results of the incubation of rat adrenal cortex cells with OP and the subsequent removal of this metal-chelating agent. The cells were incubated with Bt₂cAMP and [³⁵S]Met/Cys at 37 °C for 15 min in the presence of 2 mM OP. The cells were harvested by centrifugation at 4 °C, resuspended in incubation medium that did not contain the chelator but did contain Bt₂cAMP and CHx, and incubated at 37 °C for an additional 15 min. The same result was obtained if the chase was initiated by the addition of [³⁵S]Met/Cys (data not shown). It has been demonstrated in all systems studied that OP inhibits the metal-dependent mitochondrial protease that removes the matrix-targeting sequence. The arrows in the 0-min sample indicate the positions of migration of pp37, pp32, and pp30. Thus, pp37 is abundant in this sample, whereas pp30 and pp32 are present at very low levels. This may be due to the fact that the inhibitory action of OP did not occur immediately upon its addition or it may be due to the difficulty of obtaining a true 0-min sample after the centrifugation step. However, clearly in the 15-min sample, pp37 has decreased significantly concurrent with an increase in the levels of pp30 and its post-translational product pp30'. Thus, we find that pp37 accumulates when the cells are incubated in the presence of a metal chelator and that removal of this compound allows the amount of pp37 to decrease and the production of pp30 to occur. The shift in isoelectric point and molecular weight, i.e. pp37 is larger and more basic than pp32, is also consistent with the removal of a typical 2-10-kDa matrix-targeting sequence that lacks acidic amino acids (43, 44).

Lability of pp37 and pp32—Fig. 7 compares fluorograms of two-dimensional gels resulting from pulse-chase studies using rat adrenal cortex cells incubated at 25 °C or at 37 °C. Bt₂cAMP-stimulated cells were labeled with [³⁵S]Met/Cys for 15 min, then CHx was added, and samples taken at the times indicated. The same results were obtained if [³⁵S]Met/Cys (i.e. unlabeled) was added to stop radiolabel incorporation (date not shown). Table I shows the relative quantitation for the proteins of interest as a function of time of chase from

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**Fig. 5.** Subcellular localization of phosphoproteins pp37 and pp32. Rat adrenal cortex cells were stimulated and exposed to radiolabeled amino acids at 25 °C as for Fig. 1, except that the incubation time was 30 min. The cells were washed twice with PBS at 4 °C, then an aliquot of the cells was reserved for electrophoresis. The remainder of the cells were used for the subcellular fractionation procedure as described (31, 50). The mitochondria and the post-mitochondrial supernatant (PMS) were separated by centrifugation at 20,000 × g for 10 min. The mitochondria were washed twice in the isolation buffer. The samples were solubilized and the proteins separated by 2-D PAGE. Equal trichloroacetic acid-precipitable counts were loaded on all three gels. The proteins of interest are indicated on the fluorogram of the mitochondrial fraction. The small arrowheads in the cell and post-mitochondrial supernatant panels indicate the positions of migration of these proteins. These results were obtained in three independent experiments.

**Fig. 6.** 1,10-Orthophenanthroline inhibits the conversion of pp37 into pp30. Rat adrenal cortex cells were isolated and incubated with 2 mM OP, 2 mM Bt₂cAMP, and 1 mCi/ml [³⁵S]Met/Cys for 15 min at 37 °C, as for Fig. 1. The cells were harvested by centrifugation at 4 °C and resuspended in 37 °C KRBAG containing 2 mM Bt₂cAMP and 0.2 mM CHx. Aliquots of the cells were taken immediately after they were resuspended in the chase medium (t = 0) and 15 min later. Electrophoresis was carried as for Fig. 1. Pulse-chase studies with OP were carried out twice independently.
two to four experiments at the physiological temperature 37 °C. For pp37 the data may be fitted by first order kinetics analysis. Analysis of the pooled data in Table I by linear regression gives a straight line with a correlation coefficient of 0.96 and indicates that the half-life of pp37 is 5 min. If the data from each experiment are analyzed separately, the correlation coefficients for the four independent experiments are 0.79, 0.97, 0.98, and 0.99 and the mean half-life of pp37 calculated from these data is 5.4 ± 1.1 min (mean ± S.E., n = 4). Additionally, the half-life of pp32 at the physiological temperature is calculated to be 4.6 min from the pooled data, where the correlation coefficient of the line fitting these data is 0.97. If the data from the individual experiments are analyzed separately, the mean of the half-life for pp32 is 4.4 ± 0.8 min (mean ± S.E., n = 3). This species is present in much lower concentration than either pp37 or pp30 so that the calculations are inherently less accurate. However, the correlation coefficients for the separate lines are 0.88, 0.92, and 0.99. Thus, these proteins are converted into pp30 very rapidly at physiological temperature. The data taken at 25 °C shows that the half-life of pp37 is increased by a factor of 2–3. Additionally, in these samples the concentration of pp32 increases transiently for the initial 5 min of the chase period, then it plateaus. For the gels shown in Fig. 7, the concentration of pp32 increases by 20–30% during the first 10 min of chase. Additionally, the concentration of pp30 increases more slowly than it does at 37 °C. Thus, it is necessary to use the lower incubation temperature to observe the two higher molecular weight proteins readily, since their half-life is greatly increased by this incubation condition. These data, coupled with the structural similarities of these three proteins detected by peptide mapping (Fig. 2) and the OP-induced accumulation of pp37 shown in Fig. 6, provide further evidence for the hypothesis that pp37 and pp32 are precursors of pp30. Moreover, we find that this processing event occurs within the mitochondrion and is temperature-sensitive.

**DISCUSSION**

We have presented data in this paper demonstrating the existence of two proteins that are probable precursors of pp30. Their structural relationship to pp30 is established by the similarity of their proteolytic peptide maps to that of pp30. Additionally, we have demonstrated directly that they are phosphorylated and that they are localized to the mitochondrion. We find that lowering the incubation temperature or adding a metal chelator to the stimulated cells cause the accumulation of these proteins, without selectively inhibiting the synthesis of pp37 (data not shown). The former has been documented to slow mitochondrial import and processing (45) and the latter to inhibit the action of the metal-dependent processing protease in the mitochondrial matrix (40). Since OP causes accumulation of pp37 and removal of this chelator allows production of pp30, we can be more reasonably certain that the initial cleavage to produce pp32 from pp37 is catalyzed by the metal-dependent matrix protease (40). Determination of whether the subsequent cleavage to produce pp30 from pp32 is catalyzed by the processing protease in the inner membrane (48) or by a second protease in the matrix (38, 39) awaits further experimentation. However, since pp30 is an integral membrane protein, the former may be more likely and the sequence removed may well be a sorting sequence.

Since pp30 is produced with the same dose-response to stimulant as steroid hormone biosynthesis and begins to accumulate ~3 min after the addition of either ACTH or cAMP analogue (26-31), we can infer that its precursors appear even more rapidly and that their response to stimulant dose parallels that of steroidogenesis. Also relevant to this postulated activity is the fact that the majority of cytosolically translated proteins, entering the mitochondrion, do so via contact sites between the outer and inner mitochondrial membrane (57). In addition it has been reported that phospholipid transport occurs at these sites (58). Transit of pp37 through the contact sites could allow for transport of cholesterol from the outer membrane, where it accumulates in response to stimulant in a CHx-sensitive manner, to the inner mitochondrial membrane, where this cholesterol could be available as a substrate for the cytochrome P-450ops. This increased cholesterol concentration would, of course, produce an enhanced rate of steroidogenesis. We are currently investigating whether there is direct coupling of cholesterol transport to pp37 import.

Although the synthesis of pp30 ceases upon the addition of cytosolic translation inhibitors, pp30 is not degraded rapidly (26-31), whereas the rate of steroidogenesis returns to low,
basal levels within min (21, 22). This discrepancy was difficult to reconcile with pp30 serving as a mediator in the regulation of steroid hormone biosynthesis by peptide hormones. The data presented in this paper, demonstrating the existence of short-lived precursors of pp30 would offer an explanation for the observed lability. These precursors of pp30 are converted into mature pp30 so rapidly that it is difficult to detect them when cells are incubated at physiological temperature. Thus, these precursors have the property of “lability,” required of the postulated “labile regulatory protein.” Additionally, in our model the lability would be that of function; once the protein pp37 had been translocated with cholesterol into the mitochondrion, the precursor would be removed by the matrix protease and the resultant protein pp32 would not be able to exit the organelle. The observation that the final protein product pp30 is membrane-associated would also be consistent with its postulated function as cholesterol carrier. Thus, we have detected in rat adrenal cortex mitochondria labile proteins that may function in the regulation of adrenal corticosteroidogenesis.

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