Biosynthesis and Topogenesis of Aspartate Aminotransferase Isoenzymes in Chicken Embryo Fibroblasts

THE PRECURSOR OF THE MITOCHONDRIAL ISOENZYME IS EITHER IMPORTED INTO MITOCHONDRIA OR DEGRADED IN THE CYTOSOL *

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In chicken embryo fibroblasts pulsed with [35S]methionine, a precursor of mitochondrial aspartate aminotransferase with higher molecular weight (AM, $\approx$ 3000) was detected by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Peptide mapping of the precursor and the mature enzyme confirmed their precursor-product relationship. No precursor of the homologous cytosolic isoenzyme was found. The precursor of the mitochondrial isoenzyme is synthesized on membrane-free polysomes in the cytosol (Sonderegger, P., Jaussi, R., Christen, P., and Gehrke, H. (1982) J. Biol. Chem. 257, 3339-3345); its half-life is 30 to 60 s. The pronounced susceptibility of the precursor toward exogenous proteases contrasts the stability of the mature enzyme and thus indicates that the conformation or the quartenary structure of the protein must change concomitantly with its import into mitochondria.

Administration of the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP) to the cell cultures blocks the import of many matrix and inner membrane proteins into mitochondria. The precursor of mitochondrial aspartate aminotransferase is found to be accumulated in the cytosol. However, its steady state concentration in CCCP-treated cells exceeds the concentration in untreated cells by not more than 1 order of magnitude. During a chase, the radioactive precursor disappears with a half-life of $\approx$ 5 min without formation of mature enzyme. Thus, in CCCP-treated cells, a degradative process is limiting the accumulation of the precursor in the cytosol. When the chase is performed in the presence of cysteamine, an antagonist of CCCP, the precursor is processed to the mature enzyme. Newly synthesized cytosolic aspartate aminotransferase is not degraded.

The cytosolic and the mitochondrial isoenzymes of AA$\alpha$Tase$^1$ are homologous proteins (1-4) with almost identical folding of the polypeptide chain (5, 6). The intracellular heterotopism of the two isoenzymes has been shown to be realized both in mammals (7, 8) and birds (9) and is thought to exist in all eucaryotes. Both isoenzymes are coded for by nuclear DNA and are synthesized by cytosolic polysomes (10-12).

As a first step toward the elucidation of the mechanism of the selective import of mAATase from its site of synthesis into the mitochondrial matrix, we have analyzed, in a previous study (13), the primary in vitro translation product of the enzyme. Messenger RNA was isolated from chicken heart and translated in a rabbit reticuloocyte lysate. Mitochondrial AA$\alpha$Tase was found to be synthesized as a precursor of higher molecular weight (AM, $\approx$ 3000 (13)), whereas the molecular weight of the primary translation product of the cytosolic isoenzyme appeared to be the same as that of the mature isoenzyme isolated from chicken tissues (14). In this study, the higher molecular weight precursor of mAATase was detected in cultured chicken embryo fibroblasts, allowing the study of its destiny in intact cells.

MATERIALS AND METHODS

Most methods used in this study have been described previously (13, 14). Quantitative immunoprecipitations were performed with antisera from rabbits against SDS-denatured mAATase or CAATase (14). The antisera were noncross-reacting and monospecific. SDS-polyacrylamide gel electrophoresis of the immunoprecipitates was performed according to Laemmli (15). CCCP and bongkrekic acid were kindly donated by Dr. P. G. Heytler (DuPont & Co.), and Drs. G. W. Lijmbach and W. Berends (University of Delft), respectively. Staphylococcus aureus (Cowan strain 1) cells were kindly supplied by P. Bohni and Dr. G. Schatz (Biocenter, Basel, Switzerland). The chicken embryo fibroblasts (16) were kept at 37 °C in Eagle's basal medium ( Gibco) supplemented with 10% fetal bovine serum. For pulsing, the culture medium, usually 10 ml/Petri dish (2 x 10$^5$ cells), was replaced by 2 ml of Eagle's basal medium prepared without unlabeled methionine. For conditioning the cells, this medium contained 20 μM CCCP.

RESULTS

Occurrence of a Precursor of mAATase in Chicken Embryo Fibroblasts—When chicken embryo fibroblasts were labeled with [35S]methionine (20 μCi ml$^{-1}$) during relatively long pulses (2 h), immunoprecipitation with anti-mAATase or anti-cAA$\alpha$Tase antiserum and analysis of the precipitates by SDS-polyacrylamide gel electrophoresis gave, in both cases, only one specific band. The specific bands were located at the same molecular weight as mature mAATase or cAA$\alpha$Tase, respectively (not shown).

Portions of this paper (including part of "Results," Figs. 2, 3, 6, 8 and Table I-III) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 82 M-1174, cite the authors, and include a check or money order for $5.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

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$^1$ The abbreviations used are: AA$\alpha$Tase, aspartate aminotransferase; mAATase, mitochondrial AA$\alpha$Tase; pre-mAATase, precursor of mAATase; cAA$\alpha$Tase, cytosolic AA$\alpha$Tase; SDS, sodium dodecyl sulfate; CCCP, carbonyl cyanide m-chlorophenylhydrazone.
The amount of mAATase formed in fibroblasts is ~0.04% of the total protein synthesized. This value corresponds closely to that found after in vitro translation of chicken liver polyribosomes (14). After shorter pulses (<15 min) with at least 10-fold higher radioactivity (~0.2 mCi ml^(-1)), the band of cAATase was in an unchanged position, while, in the case of mAATase, an additional radioactive band with higher molecular weight was detected (ΔM, ~3000; Fig. 1, first lane). As expected for a precursor, the radioactivity in the band of pre-mAATase could be quantitatively chased with unlabelled methionine into the band of mAATase (Fig. 7, lane d). The half-life of pre-mAATase is estimated to be 30 to 60 s (Table 1).

Chemical cleavage of mAATase and of pre-mAATase generated very similar peptide maps (Fig. 2).

**Inhibition of Import of pre-mAATase into Mitochondria—** Treatment of chicken embryo fibroblasts with the protonophore CCCP can be expected to reduce the membrane potential of their mitochondria (see Ref. 17). At 20 μM, CCCP pre-mAATase was accumulated and no newly generated mAATase was found. The same effect was brought about by 200 μM antimycin A (Fig. 1, lane A200), most likely because antimycin A at this high dosage reduces the membrane potential (18). Antimycin A at 20 μM did not cause an accumulation of pre-mAATase (Fig. 1, lane A0) although this dose should suffice for blocking the respiratory chain (18). Similarly, other inhibitors of respiration (200 μM rotenone or 5 mM KCN), labeling of chicken embryo fibroblasts under anaerobic conditions, or the combination of these treatments with the application of inhibitors of the ADP-ATP translocator (20 μM bongkrekic acid or 30 μM carboxyatractyloside) did not affect the processing of pre-mAATase (data not shown). Cytosolic AATase showed the same molecular weight in control cells and cells treated with CCCP (Fig. 1). As shown by two-dimensional polyacrylamide gel electrophoresis, the import not only of pre-mAATase but also of many other precursors of mitochondrial proteins is completely blocked on administration of CCCP (Fig. 3).

**Intracellular Location of pre-mAATase—** Cells were pulsed and treated with CCCP 5 min after the onset of the pulse. Their homogenate contained similar amounts of label in pre-mAATase and mAATase and was fractionated by centrifugation. The postribosomal supernatant contained ~75% of the total amount of precursor (Fig. 4, lane S). The rest of pre-mAATase was recovered in the pellet (Fig. 4, lane P). Mature mAATase, however, analyzed both after protein staining and fluorography, was distributed inversely (>90% in the pellet fraction). The distribution of cAATase (~75% in the supernatant and the rest in the pellet) closely corresponded to that of pre-mAATase. Thus, in cells treated with CCCP, the bulk of pre-mAATase appears to be located in the cytosol. In accordance, pre-mAATase in unfractionated homogenates is fully susceptible to mild proteolytic digestion (Fig. 4, lanes T1 to T7).

**Difference in Structure of pre-mAATase and mAATase—** During the experiments on the intracellular location of pre-mAATase, we noticed that the precursor was exceptionally sensitive to proteolysis. It was digested at protease concentrations where cAATase and most other cell proteins were stable. In contrast, isolated mAATase proved highly resistant against trypsin (21) and other proteases. The question arose whether this difference was a consequence of a structural difference between pre-mAATase and mAATase or was due to the different conditions under which they had been exposed to proteases, i.e. in a cell homogenate and in a buffer solution, respectively. Therefore, a control experiment was performed with a homogenate in which the endogenous mAATase had been released with Triton X-100. In a second control experiment, conventionally prepared mAATase was added to a high speed supernatant containing only pre-mAATase. Both newly processed endogenous mAATase (Fig. 5A) and conventionally prepared exogenous mAATase (Fig. 5B) proved resistant against proteolyis under conditions that led to complete degradation of pre-mAATase.

**Rapid Degradation of pre-mAATase Consequent to Blocking Its Import into Mitochondria—** Treatment of the cells with CCCP decreased the rate of total protein synthesis (Table III) but, with a few exceptions, did not change the pattern of newly synthesized proteins (Fig. 3A). One of these exceptions is pre-mAATase; the labeling of its band started to decrease ~15 min after the onset of the pulse (Fig. 6). After pulses of 30 min in the presence of CCCP, the label found in pre-mAATase had not significantly increased, whereas the labeling of mAATase in control cells appeared to have increased linearly with time. After a 60 min pulse under CCCP, the labeling of the precursor was even decreased (Fig. 6), contrasting the behavior of the background bands (see also Table III). cAATase immunoprecipitated from the same untreated and CCCP-treated cells showed labeling kinetics similar to those of total cell proteins (Fig. 6 and Table III). The aberrant behavior of pre-mAATase indicates that the size of its pool is limited and reaches a steady state level within the first 15 min after the administration of CCCP. The maximum size of the precursor pool corresponds to about the 10-fold amount of that found in the control cells. The following experiments were designed to differentiate between reduction in synthesis or degradation as the cause of this limitation of the pool size.

Chicken embryo fibroblasts were treated with 20 μM CCCP for 1 h before pulsing. After this pretreatment, the cells were supplied with fresh medium again containing CCCP and [35S]methionine. This replacement of the medium restored the decreased rate of total protein synthesis (see Table III) to ~70% of that of the control. The incorporation of radioactivity into pre-mAATase during a pulse of 15 min was similar to that of the control (Fig. 7, lanes a and b). Thus, the capacity for synthesis of pre-mAATase is maintained for at least 1 h.

R. Behra, unpublished data.
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**FIG. 4. Different intracellular location of pre-mAATase and mAATase.** Twelve Petri dishes of chicken embryo fibroblasts were pulsed with 0.2 mCi ml⁻¹ of [³⁵S]methionine. After 5 min, CCCP was added to a final concentration of 20 μM and the pulse was continued for further 15 min. The cells were harvested in the cold and taken up in fractionation buffer (100 mM potassium acetate, 50 mM sodium phosphate, 50 mM sodium chloride, pH 7.6). Homogenization was performed in a volume of ~3.5 ml by 15 strokes at 1750 rpm with a Potter-Elvehjem homogenizer. Samples of this homogenate were used for subcellular fractionation or trypsin treatment as described below. The immune complexes obtained with antiserum against mAATase were precipitated with *S. aureus* cells (19, 20). Afterwards, the samples were processed for immunoprecipitation of cAAATase. Subcellular fractions and homogenates analyzed by immunoprecipitations, SDS-polyacrylamide gel electrophoresis and fluorography: P, combined pellets sedimented at 10,000 and 400,000 × g; S, supernatant (400,000 × g). The material applied to the gel was extracted from the total P and S fractions as obtained from 1 ml of homogenate. H, 0.5 ml of homogenate immediately denatured after cell disruption; H, 0.5 ml of homogenate incubated as a control for 30 min at 37 °C; T₁, T₂, T₃, each 0.5 ml of homogenate containing 0.1, 0.5, or 2.5 mg ml⁻¹ of trypsin, respectively, and incubated like H; M, standards for relative mass.

**FIG. 5. Proteolytic susceptibility of pre-mAAATase and mAATase.** A, eight Petri dishes of chicken embryo fibroblasts were pulsed with 0.2 mCi ml⁻¹ of [³⁵S]methionine. After 4 min, CCCP was added to a final concentration of 20 μM and the pulse was continued for further 16 min. The harvested cells were homogenized in ~3 ml of fractionation buffer (see legend to Fig. 4). Protease inhibitors (aprotinin, 1 μg ml⁻¹; chymostatin, 4 μg ml⁻¹, and pepstatin, 100 μg ml⁻¹; final concentrations) were added. After removal of sample H, the homogenate was made 1% in Triton X-100. Samples of 500 μl each were treated as indicated below and processed for immunoprecipitation with antiserum against mAATase. The fluorography of the SDS-polyacrylamide gel electrophoresis is shown. Treatment of samples: H, immediately denatured; H, incubated as a control for 20 min at 37 °C; T₁, T₂, containing 0.08 and 0.30 mg ml⁻¹ of trypsin, respectively, and incubated like H; P₁, P₂, containing 0.08 and 0.30 mg ml⁻¹ of papain, respectively, and incubated like H. B, eight Petri dishes of chicken embryo fibroblasts were labeled with [³⁵S]methionine for 15 min in the presence of 20 μM CCCP. From these cells, 2.5 ml of supernatant (400,000 × g) were prepared (see legend to Fig. 4). Samples of 500 μl each were treated as indicated below and processed for immunoprecipitation with antiserum against mAATase. The SDS-polyacrylamide gel electropherogram of the immunoprecipitates was stained with Coomassie blue and fluorographed. As a standard, 1 μg of unlabeled mAATase was applied onto the gel (M). One μg of native mAATase was added to each sample before the following treatment. S, incubated as a control for 30 min at 37 °C; T₁, T₂, containing 0.05 and 0.15 mg ml⁻¹ of trypsin, respectively, and incubated like S; P₁, P₂, containing 0.05 and 0.15 mg ml⁻¹ of papain, respectively, and incubated like S.
isoenzyme of AATase both on in vitro translation (14) and in the pulse cells (Fig. 1). The molecular weight of cAAATase and mAATase immunologically extracted from tissue homogenates of 7-day-old chicken embryos are the same as those of the corresponding isoenzymes from adult tissues (not shown). Thus, pre-mAATase does not represent a transient embryonic isoenzyme. In fibroblasts, under all conditions tested including only partial inhibition of the import of pre-mAATase into mitochondria and its processing to mAATase (5 μM CCCP, not shown), no immunospecific bands in addition to the bands of the precursor and the mature enzyme were detected, suggesting the absence of intermediate products.

In pulsed chicken embryo fibroblasts, the radioactivity of the precursor could be chased into mAATase (Fig. 7, lane d). The notion of a precursor-product relationship is fully supported by the similarity of the peptide maps of pre-mAATase and mAATase (Fig. 2).

**Turnover Rate and Size of Pool of pre-mAATase**—In the time period between 7 and 15 min after the onset of the pulse, the labeling of the precursor pool is in a steady state. Under the assumption that the import of pre-mAATase into mitochondria follows first order kinetics, the estimated half-life of the precursor is 30 to 60 s (Table I). This half-life is 3 to 4 orders of magnitude smaller than that of the mature enzyme, which has been found to be in the range of days in chicken liver (t1/2 = 2.8 days) and chicken heart (t1/2 = 3.8 days; see Ref. 22). The steady state concentration of pre-mAATase thus must be also 3 to 4 orders of magnitude smaller than that of the mature enzyme. The concentration of mAATase in chicken embryo fibroblasts as determined by radioimmunoassay is 85 ng/mg of protein (23).

The measured half-life of cAAATase in chicken liver and heart is the same as that of the mitochondrial isoenzyme (22). The similarity of the half-lives of the two isoenzymes seems also to apply to cultured fibroblasts where, in correspondence with a 3-fold higher content (23), the rate of synthesis of mAATase is severalfold higher than that of cAAATase (see Fig. 6, left).

**Import of pre-mAATase into Mitochondria**—In mitochondria both from Neurospora (24) and yeast (25), an energized inner membrane is required for the post-translational import of proteins. Mitochondria cannot incorporate the precursors of matrix proteins after administration of protonophores. The results presented here extend these findings to animal cells; application of high doses of CCCP or antimycin A results in inhibition of import (Figs. 1, lanes C50, and A500, and 3). pre-mAATase appears to be imported and processed post-translationally as indicated by the following experimental evidence. 1) pre-mAATase synthesized in vitro is incorporated into the matrix of exogenous mitochondria (26). 2) pre-mAATase is synthesized on free polyosomes (14). 3) A pool of pre-mAATase can be detected in untreated cells (Fig. 1, first lane). 4) pre-mAATase accumulated in CCCP-treated cells can be quantitatively chased into the mitochondria after releasing the import block (Fig. 7, lane d).

Post-translational import implies that the precursor encounters the mitochondrial envelope in folded state. The different susceptibility to proteolysis before and after processing (Fig. 5) indicates that, concomitantly with the uptake, the protein changes its conformation or its quaternary structure. Added mature mAATase has been reported not to compete with pre-mAATase for its in vitro import into mitochondria (26). Apparently, the import process depends on features of the spatial structure that are specific for pre-mAATase. The extra piece might serve not only as a recognition signal but also as an effector inducing a chain folding of the precursor dissimilar to that of the mature protein.
Rapid Degradation of pre-mAATase as a Consequence of Blocking Its Import into Mitochondria—Figs. 1 and 6 show that CCCP treatment of the fibroblasts results in an accumulation of pre-mAATase in the cytosol. Its steady state concentration increases by 1 order of magnitude, corresponding with a 10 times longer half-life (untreated cells, \( t_{1/2} \approx 0.5 \) min; CCCP-treated cells, \( t_{1/2} \approx 5 \) min). The new steady state concentration is reached within \( \pm 10 \) min after application of CCCP and is controlled by specific proteolytic degradation of pre-mAATase as indicated by its time-dependent disappearance during a chase (Figs. 1, lanes c, and 8). After the chase, no immunospecific bands with molecular weights below that of pre-mAATase have been detected. Apparently, the degradation proceeds rapidly to produce small fragments or other undetected intermediates (see below).

Evidence for a similar degradation of a higher molecular weight precursor of a mitochondrial protein in rat liver, namely carbamylphosphate synthetase, has been presented previously (27). The protease inhibitor p-aminobenzamidine was found to block the processing of pre-carbamylphosphate synthetase in rat liver explants. Under these conditions, the half-life of the precursor was 2 to 3 min. This estimate is similar to the half-life of pre-mAATase in CCCP-treated fibroblasts. For unknown reasons, p-aminobenzamidine failed to inhibit processing of pre-mAATase in both fibroblasts and cultured hepatocytes from chicken (data not shown).

It is not known what structural feature of these precursors directs them toward the degradative pathway. It is tempting to relate the degradation of pre-mAATase and pre-carbamylphosphate synthetase under the condition of blocked processing with the ATP- and ubiquitin-dependent degradative system (Ref. 28; for a review, see Ref. 29). This degradative system preferentially attacks "aberrant" proteins, i.e., proteins which are unusual in that they are denatured or otherwise modified or occur as aggregates (30). pre-mAATase is distinct both from most other cell proteins and from mAATase by its exceptional susceptibility to proteolysis (Fig. 5). It might well be recognized as an aberrant protein and thus be conjugated with ubiquitin and rapidly degraded.

The observed degradation might be a mere artifact evoked by the CCCP-treatment of the cells. Precursor molecules that are not imported and converted to the more stable mature form might undergo in aging process such as time-dependent denaturation in the cytosol, making them susceptible to degradation. However, it is also conceivable that the degradation is a physiological process. Degradation prior to import could contribute, in cooperation with the regulation of protein synthesis, to balancing the relative amounts of individual components that are imported into the organelle.

Acknowledgments—We thank Dr. Heinz Gehring and Renata Behra for valuable discussions and Ursula Bolliger for her excellent assistance in preparing the cell cultures.

REFERENCES


**Biosynthesis of Aspartate Aminotransferases**

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The biosynthesis and turnover of aspartate aminotransferases (AspATs) were studied in chicken embryo fibroblasts. The presence of the mitochondrial isozyme was either impaired or reduced in the non-cycling, control cells, while it was present in the non-cycling cells treated with the uncoupler CCCP. The AspATs were purified and separated into three forms: AspAT-I, AspAT-II, and AspAT-III. The relative activities of these forms were different in the control and CCCP-treated cells.

**Table I**

| Protein Form | Control | CCCP | Activity of Protein Form
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<tr>
<td>AspAT-I</td>
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<tr>
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The selective inhibition of AspAT-II and AspAT-III with specific inhibitors was observed, indicating that these forms are the predominant forms in the non-cycling cells. The turnover of AspATs was measured by analyzing the incorporation of labeled precursors into the proteins. The results showed that the turnover rate of AspATs was decreased in the CCCP-treated cells.

**Figure 1**

- Panel A: Amino acid incorporation into AspATs in non-cycling cells. The incorporation rate was decreased in the CCCP-treated cells.
- Panel B: Amino acid incorporation into AspATs in cycling cells. The incorporation rate was increased in the CCCP-treated cells.

**Figure 2**

- Panel A: Western blot analysis of AspATs in non-cycling cells. AspAT-II and AspAT-III were decreased in the CCCP-treated cells.
- Panel B: Western blot analysis of AspATs in cycling cells. AspAT-II and AspAT-III were increased in the CCCP-treated cells.

**Figure 3**

- Panel A: Amino acid incorporation into AspATs in non-cycling cells treated with CCCP. The incorporation rate was decreased in the CCCP-treated cells.
- Panel B: Amino acid incorporation into AspATs in cycling cells treated with CCCP. The incorporation rate was increased in the CCCP-treated cells.

**Table II**

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**Figure 4**

- Panel A: Western blot analysis of AspATs in non-cycling cells. AspAT-II and AspAT-III were decreased in the CCCP-treated cells.
- Panel B: Western blot analysis of AspATs in cycling cells. AspAT-II and AspAT-III were increased in the CCCP-treated cells.

**Figure 5**

- Panel A: Amino acid incorporation into AspATs in non-cycling cells treated with CCCP. The incorporation rate was decreased in the CCCP-treated cells.
- Panel B: Amino acid incorporation into AspATs in cycling cells treated with CCCP. The incorporation rate was increased in the CCCP-treated cells.

**Table III**

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**Figure 6**

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- Panel B: Western blot analysis of AspATs in cycling cells. AspAT-II and AspAT-III were increased in the CCCP-treated cells.

**Figure 7**

- Panel A: Amino acid incorporation into AspATs in non-cycling cells treated with CCCP. The incorporation rate was decreased in the CCCP-treated cells.
- Panel B: Amino acid incorporation into AspATs in cycling cells treated with CCCP. The incorporation rate was increased in the CCCP-treated cells.

**Table IV**

<table>
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Biosynthesis of Aspartate Aminotransferases

Half-life of pre-MAATase in CCCP-treated chicken embryo fibroblasts - Cells which had been pulsed under CCCP for 15 min were chased under CCCP for different periods of time. The radioactive in pre-MAATase immunoprecipitated from these samples was quantitated (Fig. B). The rate of decrease of the pre-MAATase immunoprecipitate is not as constant as the half-life of pre-MAATase in CCCP-treated chicken embryo fibroblasts of 5 min.

Fig. 4B - Half-life of pre-MAATase in CCCP-treated chicken embryo fibroblasts - Four Petri dishes of chicken embryo fibroblasts were pulsed with [35S]methionine (0.3 MCl/ml) for 5 min in the presence of 20 μM CCCP. One cell dish was chased with 20 μM CCCP at 0, 5, 10, 15, and 20 min. The other experimental procedures were the same as in Fig. 4A. (A) Autoradiography of the fluorograph obtained. (B) Measurement of the fluorograph by densitometry. The ordinate is the relative radioactivity and the abscissa is the chase time. pm, pre-MAATase; m, MAATase; c, CAATase.