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CELL-FREE SYNTHESIS AND CHARACTERIZATION OF HUMAN ADRENOCORTICAL PRO-ADRENODOXIN

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Poly(A^+)-RNAs were extracted from human hyperplasic adrenocortical tissue and translated in a wheat germ cell-free system in the presence of [^{35}S]-methionine. Labeled immuno-reactive adrenodoxin (ADX)-like material was immunoisolated and examined following mono and bi-dimensional electrophoretic analysis. Bovine mRNA translation products were analysed under similar conditions. While it was confirmed that bovine ADX was synthesized as a precursor of Mr 21 kDa, human pro-ADX was characterized for the first time as a somewhat larger moiety (24 kDa). On the other hand, both human and bovine mature mitochondrial ADX showed a Mr of 12 kDa. Electrophoretic study disclosed that the human, as well as the bovine pro-ADX could be resolved into several components differing by their pHi (6.5 and 6.9 for h-proADX and 5.9, 6.1 and 6.2 for b-proADX, respectively). This molecular heterogeneity might be explained by discrete disparity in the pro-adrenodoxin amino acid contents.

Adrenal cortex is a higly differentiated tissue in which hydroxylation reactions play a pivotal role in the biosynthetic pathways leading to active corticosteroid hormones (1-3). Two prominent hydroxylase systems have been actively studied in the past years, due to their potential role in the regulation of adrenocortical steroidogenic activity, i.e. cholesterol side chain cleavage (scc) and 11ß hydroxylase (11ß) activities. These two systems are located in the inner mitochondrial membrane compartment where they function as oxido-reduction cascades using reducing equivalents carried from NADPH to a specific cytochrome P-450 (P-450$_{scc}$ and P-450$_{11ß}$, respectively) via two matrix proteins, namely adrenodoxin reductase and adrenodoxin (2, 4). The iron-sulfur protein adrenodoxin (ADX) thus appears as a common required component of these systems. Adrenodoxin isolated from bovine adrenocortical tissue (b-ADX) is a 114 amino-acid protein of about 12,000 molecular weight (5-8), which is synthesized at the cytoplasmic ribosomal level as a precursor of 19 - 22,000 kDa (5,6,9,10). This pro-adrenodoxin is subsequently imported into mitochondria and concomitantly processed into its mature form (10-12). Bovine adrenodoxin has recently been cloned and the amino-acid sequence of the pro-adrenodoxin subsequently elucidated (13). It has been shown that

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adrenodoxin level in adrenocortical cells is controled by adrenocorticotropin via an increase of adrenodoxin mRNA synthesis (9). Adrenodoxin may thus be considered as a marker of adrenocortical cell differentiation.

On the other hand, nothing is known concerning the molecular properties of human adrenocortical adrenodoxin and its possible implication in pathological situations such as inherited adrenocortical steroid hydroxylase defects or adrenocortical hyperplasia and adrenal cortex carcinoma. In this work, human adrenodoxin (h-ADX) was synthesized using an in vitro translation system and human adrenocortical mRNA, and compared to its bovine counterpart (b-ADX) obtained under similar conditions. It was found that h-ADX was synthesized as a pro-protein of about 24 kDa, which could be resolved into two moieties differing by their pHi, while mature h-ADX exhibited an Mr of 12 kDa.

MATERIAL AND METHODS

Reagents :
[^{35}S]-Methionine (specific activity 1000 Ci/mmole) was purchased from the Radiochemical Center (Amersham, U.K.) and [^{3}H] Leucine (specific activity 50 Ci/mmole) from the Commissariat à l'Energie Atomique (Saclay, France). Oligo dT type T3 cellulose was supplied by Sigma (Saint Louis, MO, USA), X-RAY films (XAR-5) by Kodak and wheat germ by "Les Grands Moulins de Paris" (Paris, France). Pansorbin was purchased from Calbiochem. Molecular weight protein markers, as indicated, were provided by Pharmacia (Sweden). All the other reagents were of analytical grade.

Preparation of adrenocortical poly(A$^+$)-RNAs :
Bovine adrenal glands were obtained from the local slaughterhouse. After bisection, the medulla was carefully removed, the cortical tissue was scrapped from the capsule and immediatly frozen in liquid nitrogen. The hyperplasic human adrenal was obtained from a patient who presented a paraneoplasic cushing syndrome ; immediatly after surgical removal, the whole gland was frozen in liquid nitrogen.Total RNA was extracted and poly(A+)-RNAs were prepared by oligo-dT chromatography as previously described (14,15). RNA concentration was measured by spectrophotometry at 260 nm (1 OD = 40 µg/ml), RNA purity was assessed by the absorbance at 230-280 and 310 nm.

Cell-free translation assay :
The methods of cell-free translation was as previously described (16). The labeled aminoacids used were [^{3}H] Leucine (1.5 mCi/ml), or ^{35}S methionine (1 mCi/ml of translation incubation volume). The poly (A$^+$)-RNAs were used at 30 µg/ml in the incubation. The reaction was allowed to proceed for 60 mn at 30°C.

Immunoprecipitation :
The translation products were immunoprecipitated as described by Alexander et al. (17). The immunoglobulins G(IgG) were isolated from antiserum raised in goat against purified bovine adrenodoxin prepared according to (18).
The translation products (50 µl) were incubated for 1 h. at 37°C with 10 µl of purified IgG directed against bovine adrenodoxin. Specific anti rabbit IgG (10 µg) were then added and the mixture was incubated for 18 h. at 4°C. The mixture was incubated for 1 h. at 37°C after addition of 20 µl of Pansorbin. The precipitate was washed three times, and proteins eluted by buffer containing 2 % SDS, 0.33 mM EDTA. A second identical immuno-precipitation cycle was performed before analysis.

Electrophoretic analysis of the translation products :
The total translation products or the immunoprecipitates were analysed by one dimension electrophoresis, as described by Laemmli (19), using a 12,5 or 15 % polyacrylamide gel, as indicated. Two dimension electrophoretic analysis was performed according to O'FARELL (20). The first separation was done under equilibrium conditions (IEF 7000 v/h), and the second migration as for single dimension electrophoresis, using a 15 % polyacrylamide gel. Following one or two dimension electrophoresis, the gels were treated by sodium salicylate (21) and subjected to autoradiography.

RESULTS

1. Adrenocortical poly(A^+)-RNAs translation conditions.

The experimental conditions of the translation reaction using poly(A^+)-RNAs either of bovine or human origin and the wheat germ system were standardized to yield the highest efficiency. The translation efficiency was determined by measuring the amount of radiolabeled amino acid incorporated into the TCA-precipitable protein material, after incubation with either [^{3}H]-Leucine or [^{35}S]-Methionine. [^{3}H]-Leucine was chosen for further experiments because its use resulted in lower background as compared to [^{35}S]-Methionine, and because of its lower cost.

The translation efficiency was optimized with regard to the concentrations of K^+ and Mg^{++} present in the incubation. Optimal protein synthesis was observed for 1.7 mM K^+ and 70 mM Mg^{2+} in the case of bovine mRNAs whereas 2.0 mM K^+ and 80 mM Mg^{2+} were the best with the human mRNA preparations (not shown). These conditions were therefore used in all further experiments. Under these conditions, the amount of labeled amino acid incorporated into the protein material within 60 min. of incubation was proportional to the amount of mRNA introduced up to 30 µg/ml, in the case of both bovine and human preparations. In both cases, the overall translation efficiency was similar, i.e. it was about 10 fold that observed in the absence of added mRNA.

2. Human mRNA-directed in vitro synthesis of immunoreactive ADX material.

When the cell-free translation products resulting from incubation with human poly(A^+)-RNAs were analysed by monodimensional electrophoresis following immunoprecipitation with an anti b-ADX IgG preparation, a single labeled protein moiety was detected upon autoradiography, as illustrated in figure 1. This labeled band was not any more detected when the immunoprecipitation step was carried out in the presence of unlabeled purified bovine adrenodoxin, suggesting that the polyclonal antibody employed could indeed specifically recognize h-ADX as well as b-ADX. The human immunoreactive ADX-like material exhibited an Mr of 24 kDa, whereas similar experiments using bovine poly(A^+) mRNAs yielded a 21 kDa labeled product (not shown), in agreement with the results previously obtained by others

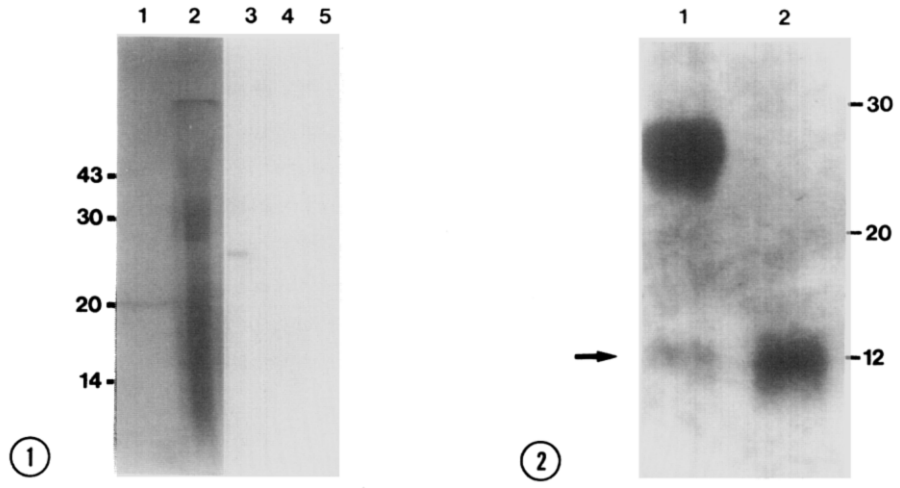

FIGURE 1 : Electrophoretic analysis of translation products of human poly(A^+)-RNAs.
Single dimension polyacrylamide gel (12,5 %) electrophoresis (19) of radiolabeled total translation products or of immunoprecipitated ADX-like material was followed by autoradiography.
- Lane 1 : total translation products, from incubation without mRNA added.
- Lane 2 : total translation products of poly(A^+)mRNA from human adrenal tissue.
- Lanes 3-4 : material resulting from immunoprecipitation of the human mRNA translation products by anti b-ADX IgG in the absence (lane 3) and in the presence (lane 4) of 10 µg of bovine mature ADX.
- Lane 5 : same as lane 4, with anti b-ADX IgG replaced by non immune rabbit IgG.

Figures on the left indicate the location of the molecular weight protein markers : ovalbumin (43 kDa) ; carbonic anhydrase (30 kDa) ; trypsin inhibitor (20 kDa) and α lactalbumin (14 kDa).

FIGURE 2 : Polyacrylamide (15 %) gel electrophoresis of mature human and bovine adrenodoxins.
- Lane 1 : material obtained following immunoprecipitation of human adrenal tissue proteins by anti b-ADX IgG.
- Lane 2 : purified bovine adrenodoxin (5 µg).

Following electrophoresis, the proteins were stained by Coomassie blue.
Figures on the right indicate the location of the molecular weight protein markers, as in figure 1. The arrow indicates the location of human and bovine mature ADX.

(5,6,9,10). However, when an adrenocortical tissue extract was treated by the b-ADX antibody preparation, the electrophoretic analysis of the immunoprecipitated material, followed by Coomassie blue staining revealed an ADX-like protein band at Mr 12,000, showing that mature h-ADX has a molecular size closely similar to that of its bovine counterpart (figure 2). It was thus concluded that human adrenodoxin appears to be synthesized in a cell-free translation system as a precursor form slighly larger in size than the bovine pro-ADX (Mr of + about 3,000), whereas a similar molecular size (12 kDa) was observed for the mature protein in both species.

3. Molecular heterogeneity of human and bovine pro-adrenodoxins.

Immunoprecipitable adrenodoxin material isolated following cell-free translation of human as well as bovine poly(A^+)-RNA preparations was analysed by two-dimensional polyacrylamide gel electrophoresis and visualized following autoradiography. As illustrated in figure 3, the 24 kDa human pro-adrenodoxin band was resolved into two components exhibiting apparent pHi of 6.9 and 6.5, respectively, the more acidic moiety being the prominent one. Both labeled components were not any more detected when the immunoprecipitation isolation step was carried out in the presence of an excess of purified unlabeled bovine ADX, suggesting that both were indeed immunologically recognized as ADX-like material.

Figure 4 illustrates the result of the two dimensional electrophoretic analysis as applied to the cell-free translation product obtained with bovine poly(A^+)-RNAs. In this case, the bovine adrenodoxin precursor (21 kDa band) was resolved into three detectable components of apparent pHi of 5.9, 6.1 (prominent) and 6.2, respectively. These data indicate that human as

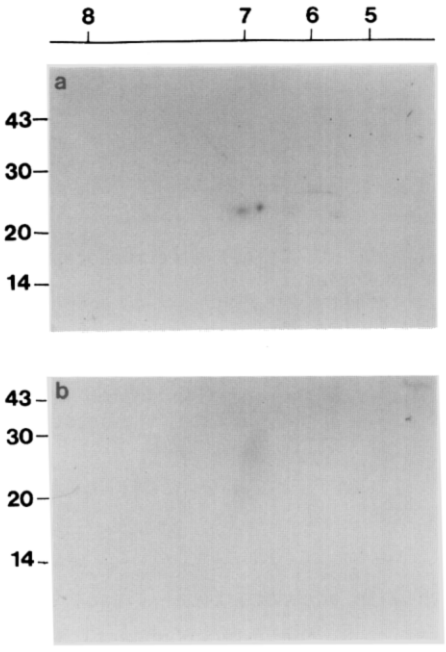

FIGURE 3 : **Two dimensional electrophoretic analysis of immunoreactive ADX-like material resulting from human mRNA cell-free translation.**
Analysis was carried out according to (20), as described in the text and followed by autoradiography.
a : immunoprecipitate obtained following treatment of the translation products with anti b-ADX IgG.
b : same as a, with immunoprecipitation in the presence of 10 µg of purified b-ADX.
Figures on the top indicate the pH gradient along the gel (first dimension) ; figures on the left show the location of molecular weight protein markers (see figure 1).

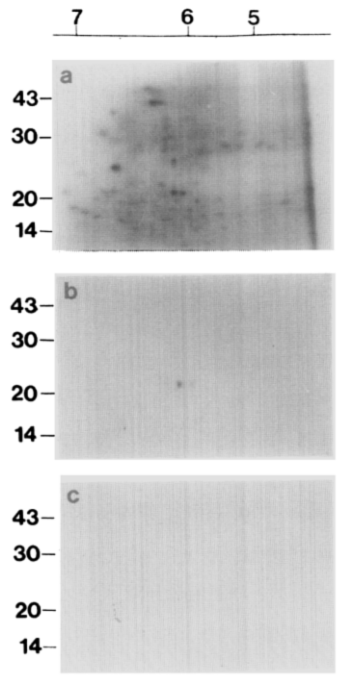

FIGURE 4 : **Two dimensional electrophoretic analysis of ADX-like material resulting from cell-free translation of bovine mRNAs.**
The analysis was performed as in figure 3. Figures on the left and on the top as in figure 3.
a : total translation products
b : immunoprecipitate resulting from treatment with anti b-ADX IgG
c : same as b, with immunoprecipitation in the presence of 10 µg of purified b-ADX.

well as bovine adrenodoxin precursors may be synthesized at the cytosolic ribosomal level as microheterogeneous mixtures of pro-ADX moieties differing by their charge in a discrete fashion.

DISCUSSION

Using a cell-free translation system and mRNA of human adrenocortical origin, it was possible in this study to characterize for the first time the human adrenodoxin system, which appeared to be synthesized as a precursor form of Mr 24 kDa, whereas the mature h-ADX exhibited a molecular size closely similar to that of its bovine counterpart, i.e. 12 kDa. This study made use of a polyclonal antibody raised against b-ADX, which apparently recognized h-ADX as well as human and bovine pro-ADX. This cross-reactivity suggests that common antigenic determinants are shared by the two proteins. This might have been expected for relatively small protein moieties (114 amino acids for b-ADX : 13) exhibiting a common redox carrier activity.

However, the bovine and the human pro-ADX translated from the corresponding mRNA under identical cell-free conditions were clearly distinguishable on the basis of their relative molecular size. The human precursor was about 3,000 daltons heavier than its bovine counterpart, which was found in this work to exhibit a Mr of 21 kDa , in agreement with values (19-22 kDa) reported by others (5,6,9,10). This size difference suggests that additional amino acids may be present in the leader sequence of the human protein, as compared to the b-proADX. It remains to be examined whether such a difference might interfere with the processing and translocation processes, required to yield the mitochondrial mature ADX form.

Another point disclosed by this study is that, following cell-free biosynthesis, both human and bovine pro-ADX appeared as heterogeneous when analysed by two-dimension electrophoresis. The components were resolved by their different pHi, in the 5.9 to 6.9 range. This heterogeneity is not likely to be due to different states of glycosylation, since the wheat germ extract used in these experiments is unable to glycosylate proteins (22). An other appealing possibility would be different states of phosphorylation of the proADX moiety, resulting in a family of protein differing by their net ionic charge. This hypothesis might be supported by the fact that b-ADX could be phosphorylated in vitro by purified cyclic-AMP dependent protein kinase (unpublished). However, incubation of the heterogeneous pro-ADX preparations with alcaline phosphatase did not modify their two-dimensional electrophoretic pattern. In addition, when purified b-ADX was incubated with the cell-free translation system in the presence of $[\gamma^{32}P]$-ATP, no incorporation of ^{32}P into ADX could be detected. These negative observations suggest that the heterogeneity of the proADX moieties biosynthesized in the cell-free system might reflect slight variation in the amino acid composition.

These observations show that adrenocortical mRNA of human origin is amenable to experimental study of its expression in a cell-free system in vitro. The present study was carried out with RNA extracted from hyperplasic human adrenocortical tissue. It is likely that normal human adrenocortical tissue will yield similar results. On the other hand, this approach might unravel anomalies of the proADX mRNA expression in pathological situations such as adrenocortical carcinoma or enzymatic defects of the corticosteroidogenic pathway in human.

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