Molecular cloning and amino acid sequence of the precursor form of bovine adrenodoxin: Evidence for a previously unidentified COOH-terminal peptide

(iron–sulfur proteins/adrenocortical steroidogenesis/mitochondrial biogenesis/nucleotide sequencing/regulation of gene expression)

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ABSTRACT   Several recombinant cDNA clones specific for the mitochondrial iron–sulfur protein adrenodoxin have been identified in a bovine adrenocortical cDNA library. One clone (pBAdx4) contains a 900-base-pair insert that includes the entire amino acid coding region of the adrenodoxin precursor protein. The amino acid sequence of mature adrenodoxin deduced from the nucleotide sequence of pBAdx4 is identical with that determined by protein sequencing except for three amide changes. The previously undetermined sequence of the adrenodoxin NH2-terminal precursor segment (58 amino acids) contains several basic residues, a characteristic feature of the precursor segment of proteins destined for mitochondria. In addition, a 14 amino acid extension is present at the COOH terminus of the mature adrenodoxin sequence. Whether this represents a COOH-terminal precursor segment is not clear. Three different adrenodoxin mRNAs are present [1.75, 1.4, and 0.95 kilobase(s) long] in bovine adrenocortical RNA. RNA from bovine corpus luteum, liver, and kidney contains transcripts that hybridize to adrenodoxin cDNA. Accumulation of adrenodoxin mRNA occurs in cultured bovine adrenocortical cells after treatment with ACTH or dibutyryl-cAMP, similar to that observed for the mitochondrial steroid hydroxylases that it services—namely, the cholesterol side-chain-cleavage cytochrome P-450 and the steroid 11β-hydroxylase cytochrome P-450.

In steroidogenic tissues, steroid hydroxylations occur in both the mitochondria and endoplasmic reticulum. In bovine adrenal cortex, for example, the cholesterol side-chain-cleavage and steroid 11β-hydroxylase enzyme complexes are in the mitochondria, while the steroid 17α-hydroxylase and 21-hydroxylase enzyme systems are in the endoplasmic reticulum (1). Mitochondrial steroid hydroxylase complexes contain a flavoprotein (adrenodoxin reductase) and an iron–sulfur protein (adrenodoxin) that act to transfer reducing equivalents from NADPH to a specific form of cytochrome P-450 [cholesterol side-chain-cleavage cytochrome P-450 ($P-450_{scc}$) and steroid 11β-hydroxylase cytochrome P-450 ($P-450_{11\beta}$) in the examples cited above]. The properties of this iron–sulfur protein (2, 3) and its interaction with $P-450_{scc}$ (4) have been characterized in detail. Mature, bovine adrenodoxin is a protein consisting of 114 amino acids and, thus, has a molecular weight of ≈12,000 (5). The mechanism by which corticotropin (ACTH), the pituitary hormone responsible for regulation of steroidogenesis in the adrenal cortex, controls the optimal in vivo levels of steroid hydroxylases (1, 6) and their related enzymes, including adrenodoxin (7, 8), has been investigated in this

laboratory. Adrenodoxin is synthesized in vitro as a precursor form of higher molecular weight (≈19,000) (9, 10). This precursor is synthesized on cytoplasmic ribosomes (11) and is subsequently incorporated into mitochondria concomitant with its processing to the mature protein with a molecular weight of 12,000 (12, 13).

To elucidate the mechanism by which ACTH regulates adrenodoxin gene expression and to investigate the nature of the precursor amino acid sequence and the processing cleavage site, we identified cDNA clones specific for bovine adrenodoxin. In so doing, we determined the nucleotide sequence of adrenodoxin cDNA and deduced the primary sequence of the adrenodoxin precursor protein. We report the existence of an additional 14 amino acids at the COOH-terminal end of adrenodoxin in addition to a 58-amino acid NH2-terminal precursor segment. We also utilized this cDNA probe to examine the tissue specificity of adrenodoxin gene expression and to assess adrenodoxin mRNA levels in bovine adrenocortical cells under a variety of conditions. We show that treatment of bovine adrenocortical cells with ACTH or cAMP analogs leads to accumulation of adrenodoxin mRNA.

MATERIALS AND METHODS

General Procedures. RNA was isolated by the procedure of Chirgwin et al. (14). Poly(A)$^+$ RNA was obtained by oligo(dT)-cellulose (P-L Biochemicals) column chromatography. Plasmid isolation, digestion with restriction endonucleases, and nick-translation were performed as described by Maniatis et al. (15). Polysomes from adrenocortical tissue were prepared by the method of Palmiter (16), and immunoselected polysomal poly(A)$^+$ RNA enriched for adrenodoxin sequences was obtained by the method of Kraus and Rosenberg (17). Radiolabeled single-stranded cDNA was prepared from this adrenodoxin-enriched poly(A)$^+$ RNA by using [α-^{32}P]dCTP (New England Nuclear; ≈800 Ci/mmol; 1 Ci = 37 GBq) (15) and was used as a screening probe. Primary monolayer cultures of bovine adrenocortical cells were prepared as described (8).

Cloning Procedures. A bovine adrenocortical cDNA library was constructed by the method of Okayama and Berg (18) as before (19). This library was screened with the cDNA probe prepared from immunoselected poly(A)$^+$ RNA. cDNA against adrenocortical 28S poly(A)$^+$ RNA isolated by sucrose density gradient ultracentrifugation (19) was used as a control. Identification of adrenodoxin cDNA clones was based

on hybrid selection of mRNA (19), followed by *in vitro* translation in a rabbit reticulocyte lysate system (New England Nuclear) and immunoprecipitation of newly synthesized adrenodoxin (7).

A second bovine adrenal cDNA library (kindly provided by D. Russell and T. Yamamoto of this institution) was prepared by a modified Okayama and Berg method (20), and a sublibrary was prepared by size-fractionating *Sal* I (P-L Biochemicals)-digested plasmids from the total library by using 0.8% low-melting agarose electrophoresis. Plasmids having insert lengths between 1 and 2 kilobases (kb) were recovered and religated with T4 DNA ligase (P-L Biochemicals) (21) and were used to transform *Escherichia coli* (strain RR1) (15). The sublibrary was screened by using the nick-translated insert of an adrenodoxin cDNA clone identified in the first library (pBAdx1).

Nucleotide Sequencing and RNA Transfer Blot Analysis. DNA fragments were labeled at the 5' ends with polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP (New England Nuclear; 3000 Ci/mmol) and were sequenced by the procedure of Maxam and Gilbert (22). In some instances, DNA fragments were subcloned into bacteriophage M13 vectors (Amersham) and sequenced by the dideoxy termination method (23) using ^{35}S–labeled deoxyadenosine 5'-[α-thio]triphosphate (New England Nuclear; 500 Ci/mmol). For blotting studies, RNA size fractionation was carried out by 1.25% agarose/formaldehyde gel electrophoresis (19) prior to transfer to nitrocellulose filter paper. Prehybridization was carried out at 65°C overnight in 10× Denhardt's solution (1× = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin) containing 4× NaCl/Cit (1× NaCl/Cit = 0.15 M NaCl/15 mM sodium citrate), 1 mM EDTA, 0.5% NaDodSO$_4$, 100 μg of sonicated salmon sperm DNA per ml, and 10 μg of poly(adenylic acid) per ml. The same conditions were utilized for hybridization with the nick-translated pBAdx4 insert (10^8 cpm/μg; 10^6 cpm/ml). After hybridization, the nitrocellulose paper was washed twice with 2× NaCl/Cit containing 0.1% NaDodSO$_4$ at 25°C for 15 min and twice with 0.1× NaCl/Cit containing 0.1% NaDodSO$_4$ for 30 min, one time at 25°C and a second time at 60°C. Filters were dried at room temperature and subjected to autoradiography.

RESULTS

Screening and Identification of Bovine Adrenodoxin cDNA Clones. In the original cDNA library, 1200 colonies were subjected to differential screening by comparing hybridization of cDNA prepared against 28S RNA (control) with that of immunoselected polysomal cDNA (adrenodoxin-enriched). Two positive clones (pBAdx1 and pBAdx2) were identified by this procedure and found to be specific for adrenodoxin by hybrid-selected translation (data not shown). The insert lengths in pBAdx1 and pBAdx2 were 600 and 550 base pairs (bp), respectively. Approximately 10,000 clones of the size-fractionated sublibrary were screened with the nick-translated insert of pBAdx1. Several putative adrenodoxin clones were identified, including pBAdx3, pBAdx4, and pBAdx5, which contain inserts 600, 900, and 1000 bp long, respectively. These clones were identified as specific for adrenodoxin by restriction endonuclease mapping and sequence analysis.

Nucleotide Sequence of Adrenodoxin cDNA. Clone pBAdx4 contains the entire amino acid coding region of the adrenodoxin precursor. Fig. 1 shows the restriction map of this clone and the sequencing strategy used to determine the complete nucleotide sequence, shown in Fig. 2. The amino acid sequence of mature adrenodoxin deduced from the nucleotide sequence is identical to that reported previously (5) except for three amide changes (Fig. 2). In addition, an NH$_2$-terminal precursor segment of 58 amino acids was
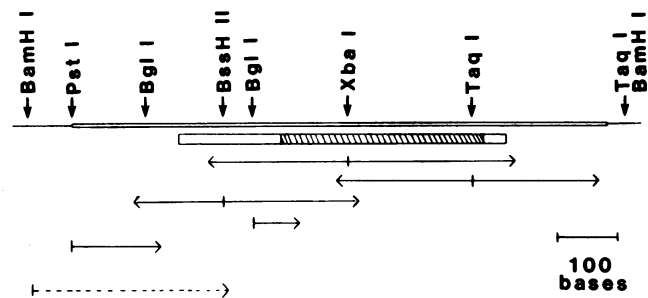

FIG. 1. DNA sequencing strategy for pBAdx4. The thin lines at the ends of the restriction map represent vector DNA. The coding sequence of the adrenodoxin precursor is represented by the box below the restriction map. The hatched region of this box represents the previously reported adrenodoxin protein sequence (5). The arrows indicate the sequencing strategy, with the labeled 5' ends being designated by the perpendicular lines. The region designated by the dashed line was determined by M13 sequencing.

present, and an additional sequence of 14 amino acids was present at the COOH-terminal end of the mature protein before a stop codon (TAA) was observed (Fig. 3). Clones pBAdx1 and pBAdx5 also contain the same sequence encoding the COOH-terminal portion of the adrenodoxin protein. Thus, the adrenodoxin precursor consists of 186 amino acids having a molecular weight of 19,787. The 5' noncoding region of pBAdx4 consists of 164 bases, and the 3' noncoding region of this clone contains 85 bases. A modified poly(A) addition signal (A-T-T-A-A-A) begins 25 bases upstream from the 70-base poly(A) sequence. The assignment of the initiator codon (Fig. 2) is based primarily on molecular weight in the absence of a stop codon in the region identified as being 5'-untranslated. The mature adrenodoxin sequence encodes 114 amino acids having a molecular weight of 12,470. By electrophoresis, this molecular weight is estimated to be 12,000 (10). The complete adrenodoxin precursor as shown in Fig. 2 has a molecular weight of 19,787. By electrophoresis, this molecular weight is estimated to be 19,000 (10). Were the sequence assigned as 5'-untranslated to be coding, the precursor would contain a minimum of 55 additional amino acids, thereby having a molecular weight of 25,382, which easily would be distinguished from 19,000.

Adrenodoxin mRNA Sizes and Tissue Distribution. Three different sizes of adrenodoxin mRNA (1.75, 1.4, and 0.95 kb) were identified in bovine adrenocortical RNA by transfer blot analysis (Fig. 4A). The differences in RNA size were found to result from the utilization of multiple poly(A) addition sites (unpublished data) as has been reported for other mRNAs (24, 25). mRNA transcripts that hybridize to adrenodoxin cDNA were also present in bovine liver, kidney, and corpus luteum (Fig. 4B). In these tissues, the mRNA sizes were either identical or similar to those in adrenal cortex.

Regulation of Adrenodoxin mRNA Levels by ACTH and cAMP Analogs. The levels of the three adrenodoxin transcripts in monolayer cultures of bovine adrenocortical cells were increased significantly by treatment with ACTH or dibutyryl-cAMP (Fig. 5). This accumulation was inhibited by treatment of cells with the transcriptional inhibitor, actinomycin D (lanes 5 and 8), or with the translational inhibitor, cycloheximide (lanes 4 and 7). The cycloheximide effect was found to be quite specific based on the fact that total transcription as measured by nuclear transcription run-off assays was the same with nuclei isolated from either control or cycloheximide-treated cells (unpublished data). Thus, the regulation of synthesis of both P-450$_{scc}$ (19) and P-450$_{11\beta}$ (26) and the protein required for their reduction, namely adrenodoxin, proceeds by similar mechanisms. The cAMP-dependent transcriptional activation of genes encoding these enzymes appears to be mediated by protein factors.

TGGCGAACGCATCAGGCCCTCTAGGGAACCCCGGCCCTGAGAGT _ 1 2 1

CCGCTGCGCCTCTCTTCTCTCCGCAGACCCGCGCGGCGTCAGCCGCCCGTACTCGCTGAGTCTGCCCTCGCGGCCGCTGCCGTGCGGCCTGCGTCGCCTTCCGTAGTTCCCCGACAGGCT _ 1

ATG GCC GCA CGC CTC CTG CGC GTC GCC TCC GCC GCC CTC GGC GAC ACG GCC GGC CGG TGG CGG CTT CTC GTG CGA CCG CGC GCG GGA GCC 9 0
Met Ala Ala Arg Leu Leu Arg Val Ala Ser Ala Ala Leu Gly Asp Thr Ala Gly Arg Trp Arg Leu Leu Val Arg Pro Arg Ala Gly Ala
                    -50   -40   -30

GGC GGC CTC CGT GGG AGC CGA GGA CCG GGC CTG GGC GGC GGC GCA GTG GCG ACG CGG ACG CTG AGC GTA TCG GGG CGA GCG CAG AGC AGC 1 8 0
Gly Gly Leu Arg Gly Ser Arg Gly Pro Gly Leu Gly Gly Gly Ala Val Ala Thr Arg Thr Leu Ser Val Ser Gly Arg Ala Gln Ser Ser
                -20   -10   -1 1

TCA GAA GAT AAA ATA ACA GTC CAC TTT ATA AAC CGT GAT GGT GAA ACA TTA ACA ACC AAA GGA AAA ATT GGT GAC TCT CTG CTA GAT GTT 2 7 0
Ser Glu Asp Lys Ile Thr Val His Phe Ile Asn Arg Asp Gly Glu Thr Leu Thr Thr Lys Gly Lys Ile Gly Asp Ser Leu Leu Asp Val
        *   10   20   30

GTG GTT CAA AAT AAT CTA GAT ATT GAT GGT TTT GGT GCA TGT GAG GGA ACC TTG GCT TGT TCT ACC TGT CAC CTC ATC TTT GAA CAG CAC 3 6 0
Val Val Gln Asn Asn Leu Asp Ile Asp Gly Phe Gly Ala Cys Glu Gly Thr Leu Ala Cys Ser Thr Cys His Leu Ile Phe Glu Gln His
                        40   50   60

ATA TTT GAG AAA TTG GAA GCA ATC ACT GAT GAG GAG AAT GAC ATG CTT GAT CTG GCA TAT GGA CTA ACA GAT AGA TCG CGG TTG GGC TGC 4 5 0
Ile Phe Glu Lys Leu Glu Ala Ile Thr Asp Glu Glu Asn Asp Met Leu Asp Leu Ala Tyr Gly Leu Thr Asp Arg Ser Arg Leu Gly Cys
                    70   *   *   80   9

CAG ATC TGT TTG ACA AAG GCT ATG GAC AAT ATG ACT GTT CGA GTA CCT GAT GCC GTG TCT GAT GCC AGA GAG TCC ATT GAT ATG GGC ATG 5 4 0
Gln Ile Cys Leu Thr Lys Ala Met Asp Asn Met Thr Val Arg Val Pro Asp Ala Val Ser Asp Ala Arg Glu Ser Ile Asp Met Gly Met
                        100   110   120

AAC TCC TCA AAG ATA GAA TAA ATAGGGAATATTTCACAACATTTTACCCTATTTTTATAATTATTATTTCTTAATGTA**ATTAAAT**GAGAACATGGATGAATGGAAAAAAAAAA 6 5 3
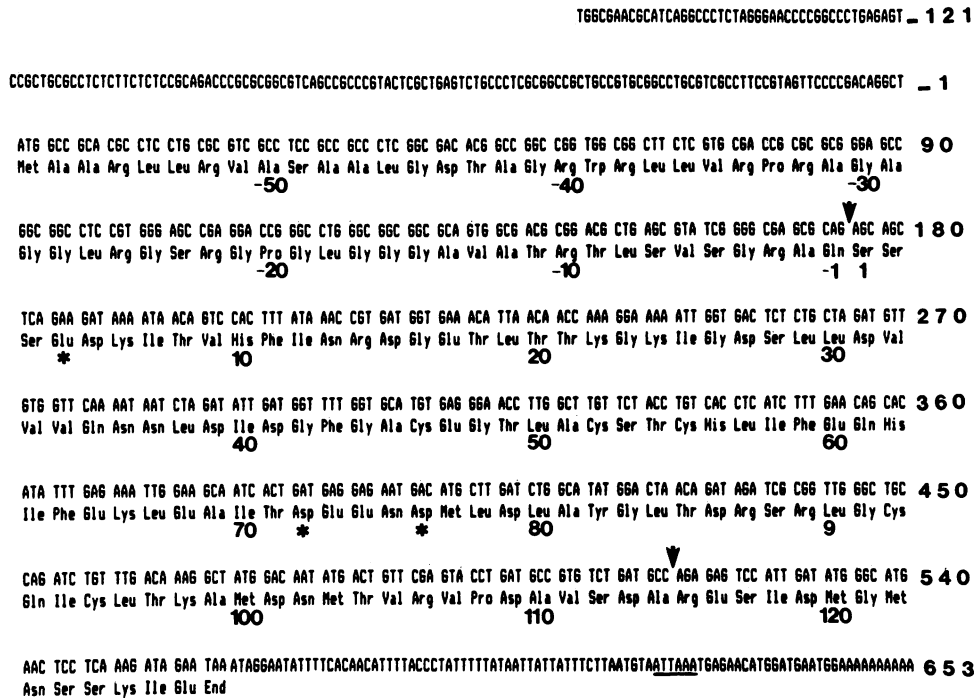Asn Ser Ser Lys Ile Glu End

FIG. 2. The nucleotide sequence of pBAdx4 and the amino acid sequence deduced from this data. The NH₂-terminal and putative COOH-terminal cleavage sites are indicated by arrows. The amide differences between the deduced primary sequence and that determined previously (5) are indicated by asterisks. The putative poly(A) addition signal on pBAdx4 mRNA is underlined.

DISCUSSION

The amino acid sequence of the bovine adrenodoxin precursor protein deduced from the nucleotide sequence of pBAdx4 contains three interesting features. First, the sequence of mature adrenodoxin is identical to that determined by protein sequencing (5) with the exception of three amide changes noted in Fig. 2. The protein is acidic, containing 21 acidic amino acids and 9 basic amino acids. The NH₂-terminal

precursor sequence consists of 58 amino acids, 10 of which are basic and 1 of which is acidic. A common feature of the prepiece of cytoplasmic precursors of mitochondrial proteins is their basic character (27). Thus, when the adrenodoxin prepiece is cleaved following import into the mitochondrion, the protein becomes more acidic. The functional significance of this change in charge may be related to the electrostatic nature of the adrenodoxin–P-450 and adrenodoxin–adrenodoxin reductase interactions. In mature adrenodoxin, the

FIG. 3. Sequence of the COOH-terminal extension determined from a 200-bp *Taq* I–*Taq* I fragment (see Fig. 1). An autoradiogram from 8 M urea/15% PAGE is shown. The three codons at the bottom represent the COOH terminus of the previously known mature adrenodoxin sequence (5); the remainder of the sequence is that of the COOH-terminal extrapeptide. The arrow indicates the putative COOH-terminal cleavage site.
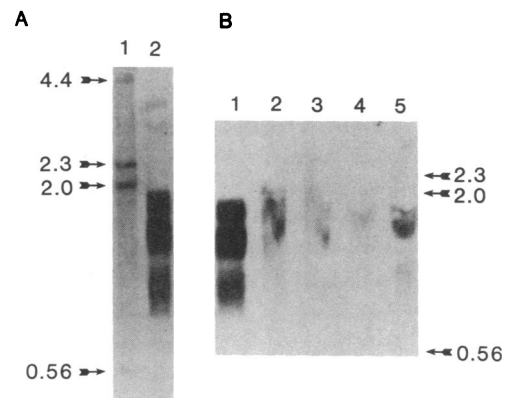

FIG. 4. Size and tissue distribution of adrenodoxin mRNA. RNAs from several tissues were run on agarose/formaldehyde gel; after transfer to nitrocellulose paper, the RNAs were hybridized with the nick-translated pBAdx4 insert (*Bam*HI–*Bam*HI) (10⁸ cpm/μg, 10⁶ cpm/ml) by using [α-³²P]dCTP (New England Nuclear; ≈800 Ci/mmol). Lanes in *A*: 1, size markers [0.2 μg of phage λ DNA (*Hind*III digest) hybridized with nick-translated phage λ DNA; sizes are shown in kb]; 2, 2 μg of poly(A)⁺ RNA from bovine adrenal cortex. Lanes in *B*: 1, 3 μg of poly(A)⁺ RNA from bovine adrenal cortex; 2, 20 μg of poly(A)⁺ RNA from bovine liver; 3, 20 μg of poly(A)⁺ RNA from bovine kidney; 4, 40 μg of total RNA from human fetal adrenal gland; 5, 40 μg of total RNA from bovine corpus luteum. Final wash conditions in *A* and *B* were 0.1× NaCl/Cit containing 0.1% NaDodSO₄ for 30 min at 60°C.
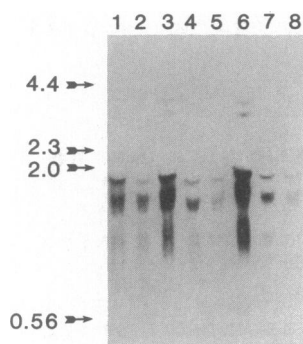
FIG. 5. Transfer blot analysis of the RNAs from cultured bovine adrenal cortical cells maintained under various conditions. Lanes: 1, 20 μg of total RNA from bovine adrenal cortex (2.7); 2, 20 μg of total RNA from control (untreated) cells (1.0); 3, 20 μg of total RNA from cells treated with 1 μM ACTH for 24 hr (19.4); 4, 20 μg of total RNA from cells treated with 1 μM ACTH and cycloheximide at 20 μg/ml for 24 hr (1.0); 5, 20 μg of total RNA from cells treated with 1 μM ACTH and actinomycin D at 1 μg/ml for 24 hr (0.04); 6, 20 μg of total RNA from cells treated with 1 mM dibutyryl-cAMP for 24 hr (30.2); 7, 20 μg of total RNA from cells treated with 1 mM dibutyryl-cAMP and cycloheximide at 20 μg/ml for 24 hr (1.5); 8, 20 μg of total RNA from cells treated with 1 mM dibutyryl-cAMP and actinomycin D at 1 μg/ml for 24 hr (0.15). The hybridization procedure was identical to that described in Fig. 4 as were the molecular weight markers, also shown in kb. The lanes were scanned by densitometry, and the sum of the three bands for each lane is given in parentheses. These values have been normalized based on control cells (lane 2) = 1.0.

sequence between the two pairs of cysteine residues implicated in iron–sulfur-center formation (Cys-52 and Cys-55; Cys-92 and Cys-95; see ref. 5) contains nine acidic residues. Specific modification of three of these residues results in diminished interaction of adrenodoxin with adrenodoxin reductase and P-450$_{scc}$ (28, 29). Thus, the removal of the basic precursor segment may expose the above acidic region of mature adrenodoxin and allow formation of functional P-450$_{scc}$ and P-450$_{11\beta}$ enzyme systems.

Second, comparison of homology between the adrenodoxin precursor segment and that of bovine P-450$_{scc}$ (30) (alignment of the 13 NH$_2$-terminal residues of the adrenodoxin precursor with the 12 NH$_2$-terminal residues of the P-450$_{scc}$ precursor) reveals sequence identity with 9 of the 13 NH$_2$-terminal adrenodoxin residues (Fig. 6A). This sequence is probably not important in targeting the proteins to steroidogenic mitochondria because P-450$_{scc}$ is taken up and processed only by such mitochondria, whereas adrenodoxin can be imported and processed by all mitochondria (12). Homology between this region of the P-450$_{scc}$ sequence and the NH$_2$-terminal precursor sequences of yeast cytochrome *c* peroxidase and elongation factor Tu has been noted also

(30). On the other hand, adrenodoxin and P-450$_{scc}$ show little sequence homology in the region of their cleavage sites (Fig. 6B). This finding is consistent with the observation that a matrix fraction from heart mitochondria can cleave the adrenodoxin precursor but not the P-450$_{scc}$ precursor (12). To date, no consensus amino acid sequence at the cleavage site of mitochondrial precursor proteins has been identified.

Third, the presence of an additional amino acid sequence at the COOH-terminal end of the adrenodoxin precursor is unexpected. In reporting the amino acid sequence of mature adrenodoxin (5), it was noted that carboxypeptidases A and B failed to liberate any amino acids from adrenodoxin but that alanine was liberated in 60% yield as the COOH-terminal residue by hydrazinolysis. Thus, we deduce that the additional COOH-terminal peptide was not found in the original study because (*i*) it was removed proteolytically during adrenodoxin purification, or (*ii*) it is a COOH-terminal precursor extrapeptide that is removed upon uptake and processing of the adrenodoxin precursor by mitochondria. In this context, two electrophoretically distinct forms of adrenodoxin have been observed in bovine adrenal cortex and corpus luteum (31). If *ii* were correct, adrenodoxin would provide the first example of COOH-terminal extrapeptide associated with a mitochondrial precursor encoded by nuclear genes. In chloroplasts, a COOH-terminal extension has been observed for a protein encoded by chloroplast DNA (32), and in secretory proteins such as procollagen and proglucagon, proteolytic processing occurs at both the NH$_2$ and COOH ends (33, 34).

The presence of adrenodoxin mRNA in corpus luteum (Fig. 4B) was expected as this tissue contains a P-450$_{scc}$ enzyme complex, and adrenodoxin from this tissue appears to be identical with that from adrenal (31). The appearance of liver and kidney RNA transcripts that hybridize to adrenodoxin cDNA is more interesting. Both tissues contain mitochondrial forms of P-450: the liver, a steroid 26-hydroxylase required for bile acid synthesis (35); and the kidney, a 25-hydroxyvitamin D 1α-hydroxylase (36). Both of these enzyme activities can be reconstituted with adrenodoxin, and a similar iron–sulfur protein has been purified from kidney mitochondria (37). The less-intense hybridization signal for liver and kidney relative to adrenal cortex may result from a smaller number of transcripts or from lack of strong homology of such transcripts for adrenodoxin cDNA, or from both. Nevertheless, a protein similar to adrenodoxin is present in both liver and kidney, thus explaining why these tissues have the capacity to process the adrenodoxin precursor (12). Human fetal adrenal RNA also contains RNA transcripts that hybridize to bovine adrenodoxin cDNA, indicating homology between these two species.

We have shown previously that the synthesis of the steroid hydroxylases and related enzymes is induced by ACTH in
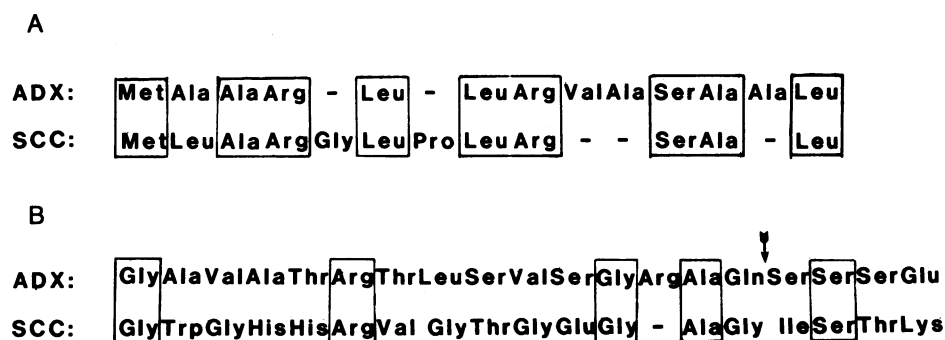
A

B

FIG. 6. Comparison of selected amino acid sequences between the adrenodoxin and the cytochrome P-450$_{scc}$ prepieces. (*A*) N-terminal sequences of the two precursors, (*B*) sequence of the two precursors around their cleavage sites which are indicated by arrow. The P-450$_{scc}$ sequences are taken from ref. 30.

primary cultures of bovine adrenocortical cells (6) and that this induction is mediated via cAMP (8). Furthermore, treatment of primary cultures of bovine adrenocortical cells with ACTH or dibutyryl-cAMP results in an accumulation of mRNA specific for $P-450_{scc}$ (19) and $P-450_{11\beta}$ (26). As shown in Fig. 5, the same is true for adrenodoxin, a component of both the cholesterol side-chain-cleavage and steroid 11β-hydroxylase enzyme complexes.

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