Isolation and Characterization of the cDNAs Encoding Two Isoforms of Subunit C1x of Bovine Cytochrome c Oxidase*

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The smallest subunit of bovine cytochrome c oxidase (C1x or VIII in different nomenclatures) occurs in two isoforms, a heart (H) form and a liver (L) form. The cDNAs for both of these forms have been isolated and sequenced. The cDNA for the H form encodes a protein 70 amino acids long with a 24-residue presequence and a mature polypeptide of 46 amino acids; that of liver encodes a protein of 69 amino acids, a 25-residue presequence and a mature polypeptide of 44 amino acids. The leader sequences of the H and L forms are 40% homologous with an abundance of positively charged residues but no negatively charged amino acids. These features are typical of polypeptides targeted to the mitochondrion for processing in the matrix space.

The homology of the two isoforms is 52% in the mature subunit with most of the differences occurring in the N-terminal hydrophilic domain of the protein. Evidence has been obtained of polymorphisms of both the H and L forms of the subunit.

Protein chemical analyses show that the H isoform is the predominant if not the exclusive form of subunit C1x in heart and skeletal muscle tissue. The L form is the predominant form in liver, kidney, and brain. Northern analyses, using cDNAs to the two forms to screen whole cell RNA preparations, show that the transcript of the H isoform is present in heart and skeletal muscle but not in other tissues examined. The mRNA of the L form was found in brain, kidney, and liver and also in heart and skeletal muscle. These results indicate that the synthesis of the H isoform of C1x is controlled transcriptionally while the L form is under post-transcriptional regulation at least in heart and muscle tissue.

Cytochrome c oxidase, the terminal component of the mitochondrial respiratory chain, transfers electrons from reduced cytochrome c to molecular oxygen, coupling this reaction to the generation of a proton gradient across the mitochondrial inner membrane (reviewed in Refs. 1 and 2). In mammals, the enzyme contains 13 different polypeptides, 2 hemes and 2 copper atoms as prosthetic groups, along with a zinc atom and possibly an additional copper of unknown function (2-4). The three largest polypeptides of cytochrome c oxidase are coded for by mtDNA (5). These mitochondrial synthonsed subunits are the functional core of the enzyme, acting as apoproteins for the prosthetic groups, and contributing both the electron transfer and proton pumping activities (2, 6, 7). The remaining subunits are nuclear coded, each made on free ribosomes, and targeted to the mitochondrion by signals often contained in an N-terminal extension which is cleaved to yield the mature subunit (reviewed in Refs. 8 and 9).

The function(s) of the nuclear coded subunits is unclear. Among possible roles for these subunits are regulation of electron transfer rate, Ca2+ translocation, control of proton pumping efficiency, as well as regulation of biogenesis of this enzyme and even other components of the respiratory chain (3).

It is now evident that mammalian cytochrome c oxidase occurs in different isoforms. This was first indicated in studies of Kadenbach and colleagues (10, 11) which showed the different migration of selected subunits under highly resolving conditions of NaDodSO4-polyacrylamide gel electrophoresis. More recently, Kuhn-Nentwig and Kadenbach (12) have compared the immunoreactivity of cytochrome c oxidase from different adult and fetal tissues and concluded that in rat all of the nuclear coded subunits have two or more isoforms.

We have been studying the tissue specificity of bovine cytochrome c oxidase, where the available evidence is that only three subunits have isoforms, ASA, CVI1, and C1x in our nomenclature (13). One form of these three subunits, the H form, first identified in heart tissue, is also found in skeletal muscle; the second form (L), first characterized in liver, is also present in brain and kidney.

The cDNAs for a number of the genes of cytochrome c oxidase have been isolated (e.g. Refs. 14-18). In most cases only a single gene has been characterized for each subunit. The exception is ASA (V1a) for which Schlerf et al. (17) have isolated different genes from heart and liver cDNA libraries of rat. Here we report the isolation of two different genes for C1x from beef, one coding for the H form and the other for the L form of this subunit. The deduced amino acid sequences of the two forms of C1x are compared and the cDNAs are used to examine the expression of isoforms of this subunit in different tissues.

EXPERIMENTAL PROCEDURES

Materials—All chemicals used were of the highest purity available. Restriction enzymes and T4 DNA ligase were from Boehringer-Mannheim, and polynucleotide kinase was from either International Biotechnology Inc. (IBI) or Promega Corp. Bacteriophage T7 or SP6 RNA polymerase was from Promega Corp. Radiochemicals [α-35S]Pur, a pyrimidine; Pur, a purine; bp, base pairs.

1 The abbreviations used are: NaDodSO4, sodium dodecyl sulfate; Pyr, a pyrimidine; Pur, a purine; bp, base pairs.
Sodium phosphate, pH 6.5) and transferred to nylon membranes. Oligonucleotides were synthesized corresponding to the complement of the coding strand. Hybridization was performed by selective hybridization as previously detailed (15) except hybridization with the radiolabeled probe was performed at 34 °C with a stringent final wash at 30 °C. For the isolation of the cDNA encoding subunit CIX, a λgt11 library containing bovine liver cDNAs was purchased from Clontech. Escherichia coli strain Y1090 was infected with bacteriophage to obtain approximately 50,000 plaques/15-cm plate. Phage were transferred to nylon filters (Du Pont-New England Nuclear colony/plaque screen) and DNA immobilized by standard techniques (19). Preparation of the radiolabeled oligonucleotide probe using either 3000 Ci mmol⁻¹ or 7000 Ci mmol⁻¹ [γ-32P]ATP and hybridization were performed essentially as described previously (18) except for a hybridization temperature of 37 °C and a final stringent wash at 34 °C. After exposure to Kodak XAR-5 film at −70°C for 1–2 days, plaques which bound the probe were purified and DNA phage isolated after phenol extraction of Cε1I-banded λ particles (19). The cDNA insert was removed by Eco1014 endonuclease digestion and ligated to vector plasmid SK (Stratagen) to facilitate DNA sequence analysis.

Both CIX-encoding cDNA clones were sequenced by the chain-terminating dideoxynucleotide method of Sanger et al. (20), incorporating either [α-32P]dATP or [γ-32P]ATP, and sequences were confirmed by analysis of both strands. Longer cDNA clones encoding both isoforms were isolated by selective hybridization with radiolabeled RNA transcripts from the original clones. Transcripts were synthesized in vitro from phage Sp6, T3, or T7 promoters available in the DNA-carrying vectors pGEM-3Z or pBluescript SK (21).

RNA Isolation and Northern Blot Analysis—Total cellular RNA was isolated from approximately 4 g of bovine heart, kidney, liver, brain, and skeletal muscle tissue by the method of Chirgwin et al. (22). Aliquots (20 μg) were denatured for 10 min at 65 °C in the presence of 50% formamide, 2 M formaldehyde and separated by electrophoresis through 1.5% agarose, 26 M EDTA, pH 7.8, buffer. Resolved RNA species were visualized after staining with ethidium bromide. RNA was immobilized by baking the filter at 60 °C for approximately 1 h.

For probing the RNA blots, [32P]-labeled antisense RNA transcripts of both isoforms were synthesized in vitro by the phage T7 or SP6 RNA polymerase. Hybridization was performed at 55–60 °C for 16–20 h in 50% formamide, 2 M formaldehyde and separated by electrophoresis through 1.5% agarose, 2 M formaldehyde gel support in 20 mM Hepes, 1 M EDTA, pH 7.5, buffer. Resolved RNA was visualized by ethidium bromide staining (33 μg ml⁻¹ in 10 M sodium phosphate, pH 6.5) and transferred to nylon membranes (Nytran, Schleicher & Schuell) using 20 X SSPE (3.6 M NaCl, 200 M Na2HPO4, 200 M NaH2PO4, pH 7.7, 20 mM EDTA). RNA was immobilized by baking the filter at 60 °C for approximately 1 h.

RESULTS

The cDNAs for the heart and liver forms of CIX were cloned from a bovine heart and bovine liver library, respectively, using mixed antisense oligonucleotides made against the published protein sequence data except at two positions. The cDNA sequence differs from the published protein sequence by encoding an Arg instead of a Lys at position 7. This is a polymorphism. In the course of isolating and sequencing CIXH in topology studies, we have observed both Arg and Lys at cycle 7 when hearts from single animals were used and we have found both Arg and Lys at this position when the subunit was sequenced from cytochrome c oxidase preparations purified from several hearts together.

The sequence of the cDNA for the liver form of subunit CIX (subunit CIXL) is given in Fig. 2 along with its deduced amino acid sequence. Subunit CIXL is also a precursor with an N-terminal leader sequence predicted to be 25 amino acids long and containing several positively charged residues but no negatively charged residues. The deduced amino acid sequence of the polypeptide coded for by the cDNA below, differs from the published protein sequence by encoding an Arg instead of a Lys at position 7. This is a polymorphism. In the course of isolating and sequencing CIXH in topology studies, we have observed both Arg and Lys at cycle 7 when hearts from single animals were used and we have found both Arg and Lys at this position when the subunit was sequenced from cytochrome c oxidase preparations purified from several hearts together.

Fig. 1. cDNA and protein sequence data for the CIX heart isofrm (CIXH). The predicted N-terminal precursor is boxed and putative polyadenylation signal underlined. The asterisk designates the in-frame stop codon.

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The sequence of the cDNA for the liver form of subunit CIX (subunit CIXL) is given in Fig. 2 along with its deduced amino acid sequence. Subunit CIXL is also a precursor with an N-terminal leader sequence predicted to be 25 amino acids long and, like the heart form, containing several positively charged residues but no negatively charged ones. The deduced amino acid sequence from the cDNA agrees with the partial protein sequence data except at two positions. The cDNA data indicate an Asp at residue 14 while the protein sequence data reveal a Glu at this position. We have seen an Asp rather than a Glu in protein sequencing in 3 of 7 samples of this subunit isolated from liver or brain cytochrome c oxidase (different animals), indicating that the variation at residue 14 is another polymorphism.

The second discrepancy between the deduced sequence from cDNA and protein sequence data is at residue 25 which was tentatively assigned as an Asp (13)
Sequences of Isoforms of Clx of Beef Cytochrome Oxidase

CGTTAATGTCTGTGCTGACTCCACTGCTGTTGAGAGGCCTGACAGGCCCTGCCCGGCGGC

MSVLTPLLLRGLTGPARR

TCCCAGTGCCGCGTGCCCAGATACATTCCAAGCCGCCGCGGGAGCAGCTCGGGACCATGG

NCOl LE'VPRAQIHSKPPREQLGTM

ATATCGCCATTGGGCTCACCTCCTGCTTCCTGTGTTTCCTCCTGCCTTCCGGCTGGGTCC

DIAIGLTSCFLCFLLPSGW "

TGTCACACATGGAGAACTACAAGAAGCGGGAGTGATGGGAGCTGTCCTCTCCCTCGCCCC

L s H M ENYKKRE'

GAGACCTGACCACCCCGGCCTTTTCCTGATCATGTCTGCTGCATTCCTGGCCGGCCTCCC

301 CTTGACCACGTCCTTCTGTTACAGTGACCTCTTCTGCAATCATGACGTCTTGATTTCTCC

361 ACGGTGACATCCTGGGACCACATGTATCTGTTTATAAGGCCCTGCTCAGTAGGGCCTCCC

TTGCAACAATAAAGTCTATTTAAAGCGTGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

FIG. 2. cDNA and protein sequence data for the Clx liver isoform (Clxl). The predicted presequence isboxed. Both the NcoI recognition site and polyadenylation signal are underlined. The asterisk designates the in-frame stop codon.

FIG. 3. Comparisons of protein sequences of the pre- and mature subunit Clx isoforms from several species. A, bovine heart and liver isoforms. Polymorphisms are illustrated by the inclusion of both relevant residues. B, mature protein sequence of subunit Clx from bovine liver, rat liver, and human heart. The human heart Clx sequence was obtained by protein sequencing analysis (29), the rat liver sequence predicted from the cDNA clone (16) and the bovine liver in part by protein sequencing (13) and cDNA analysis (this paper). C, predicted N-terminal presequences from the two bovine isoforms and the human subunit Clx (25). Percentage homologies are detailed in the text.

but is a Cys based on the cDNA sequence. In this case the discrepancy probably represents a protein sequencing error.

Fig. 3A compares the protein sequences of the two isoforms of Clx. The leader sequences show 40% homology while the mature proteins are homologous in 52% of residues. Part B compares the deduced amino acid sequences of the liver form of Clx from bovine liver, rat liver, and human heart. The human heart Clx sequence was obtained by protein sequencing analysis (29), the rat liver sequence predicted from the cDNA clone (16) and the bovine liver in part by protein sequencing (13) and cDNA analysis (this paper). C, predicted N-terminal presequences from the two bovine isoforms and the human subunit Clx (25). Percentage homologies are detailed in the text.

The 304-bp cDNA encoding subunit ClxNH was used to screen whole cell RNA preparations from several bovine tissues by Northern analysis. This probe hybridized to an approximately 480-nucleotide RNA in heart and skeletal muscle preparations but failed to bind to RNA isolated from liver, brain, or kidney (Fig. 4A). The 480-bp cDNA encoding the liver form of subunit Clx was also used in Northern analysis. Surprisingly, this probe hybridized to many RNA species over a wide size range even though the hybridization and washing steps were conducted at relatively high stringency. Increasing the hybridization temperature to 67 °C did not appreciably reduce the nonspecific signals. To test the possibility that the long 3'-untranslated region was responsible for the low specificity of hybridization, the 480-bp cDNA was cut with NcoI to generate a 120-nucleotide (nucleotides 1-120) fragment encoding the N-terminal leader sequence and the first 13 amino acids of the mature protein sequence. A probe from this truncated cDNA, devoid of any 3'-untranslated sequence, hybridized strongly to an RNA species of around 690 nucleotides in skeletal muscle, heart, liver, brain, and kidney total RNA preparations (Fig. 4B). The amount of this L form transcript was higher in kidney and brain than in liver, probably in proportion to the amount of mitochondria present in these different tissues (see also results in Refs. 14 and 15).

The results of these Northern analyses strongly suggest that the gene for subunit ClxNH is transcribed only in muscle cells (heart and skeletal muscle) while the gene for the L form of this subunit is transcribed in all tissues.

Another RNA species of approximately 400 nucleotides was also detected, although relatively more weakly, with the truncated ClxNH probe. The origin of this species is unknown but it is noteworthy that Zeviani et al. (14) have reported detection of a smaller, 300-nucleotide poly(A) RNA on Northern blots using a cytochrome c oxidase subunit IV probe. They found the majority of the homology to be located in the 5'-untranslated and presequence region of this subunit suggesting that the smaller species may be generated during processing or by degradation of the message.

Our previous studies had shown the different migration of the two isoforms of Clx in heart and liver cytochrome c oxidase (13). However, these data did not rule out the presence of
Fig. 5 shows the low molecular weight region of a highly resolving gel in which purified cytochrome c oxidase from heart, brain, skeletal muscle, liver, and kidney is compared. Samples were loaded in amounts to maximize detection of the two forms of CIX without compromising the resolution between the low molecular weight polypeptides. No evidence of the H form was seen in liver, brain, or kidney and there was no band in the position of the L form in heart or skeletal muscle.

The above gel electrophoresis experiments were complemented by sequence analysis. A companion gel to that shown in Fig. 5 was electrotransferred to Immobilon and a region of the blot broad enough to contain both isoforms, if present, was subjected to sequencing. The sequence of the Crx region in different tissue. It was important therefore to check for the presence of small amounts of H form in liver and vice versa.

Both isoforms, one in high amount, the other in low amount in different tissue. It was important therefore to check for the presence of small amounts of H form in liver and vice versa. Fig. 5 shows the low molecular weight region of a highly resolving gel in which purified cytochrome c oxidase from heart, brain, skeletal muscle, liver, and kidney is compared. Samples were loaded in amounts to maximize detection of the two forms of CIX without compromising the resolution between the low molecular weight polypeptides. No evidence of the H form was seen in liver, brain, or kidney and there was no band in the position of the L form in heart or skeletal muscle.

The sequence of the CIX region in heart gave a single amino acid in each cycle, corresponding to the H form with no indication of amino acids for the L form at cycles 2, 3, and 6 where the two sequences are different. Similarly, the sequence of CIX from liver gave the amino acids expected of the L form with no evidence of residues of the H form in the relevant cycles.

DISCUSSION

Several of the subunits of cytochrome c oxidase appear to occur in two (or more) isoforms in some mammals, including subunit CIX (3,13). In this study, the cDNAs encoding the two isoforms of bovine CIX were cloned. A comparison of these cDNAs and of the deduced protein sequences of the two isoforms shows several interesting features. The cDNA for the heart isoform is considerably smaller than that for the liver isoform. Similarly, the mRNA for the H form is smaller than the L form (480 cf. 690 nucleotides). Most of this difference is accounted for by the presence of a (237+ nucleotides) long 3'-untranslated sequence in the liver isoform compared with a 70-nucleotide 3'-untranslated sequence in the heart form. The 3'-untranslated region of the liver isoform is as highly conserved as the protein coding region, as highly conserved as the protein coding region, suggesting an important role for this region in transcriptional or translational regulation of this subunit. Other enzymes of energy metabolism occur in muscle-specific and non-muscle (i.e. liver, brain, kidney) isoforms (26,27). Sakoda et al. (28) have recently cloned the cDNAs for the isoforms of human phosphoglycerate mutase and find that the M (muscle) isoform has only a short (37 bp) 3'-untranslated region while the heart form. The 3'-untranslated region of the liver isoform is considerably smaller than that for the liver isoform. Similarly, the mRNA for the H form is smaller than the L form (480 cf. 690 nucleotides). Most of this difference is accounted for by the presence of a (237+ nucleotides) long 3'-untranslated sequence in the liver isoform compared with a 70-nucleotide 3'-untranslated sequence in the heart form. The 3'-untranslated region of the liver isoform is as highly conserved as the protein coding region, suggesting an important role for this region in transcriptional or translational regulation of this subunit. Other enzymes of energy metabolism occur in muscle-specific and non-muscle (i.e. liver, brain, kidney) isoforms (26,27). Sakoda et al. (28) have recently cloned the cDNAs for the isoforms of human phosphoglycerate mutase and find that the M (muscle) isoform has only a short (37 bp) 3'-untranslated region while the heart form.

The cDNA for the heart isoform of CIX encodes a precursor of 70 amino acids and a mature protein of 46 residues. The cDNA for the liver isoform encodes a precursor protein of 99 amino acids and a mature polypeptide of 44 residues. The sequence homology between the heart and liver isoform is 52%. Much of the difference in the two sequences occurs in the N-terminal extrinsic domain of the polypeptide. The long hydrophobic stretch, thought to span the mitochondrial inner membrane (residues 15-38), has 15 conserved residues which may provide a conserved interface for interaction with other transmembrane subunits.

The difference in sequence of the isoforms of CIX is greater than the species variability. Thus the homology between bovine and rat is 77% and between bovine and human is 64%. The homologies between species for CIX are the lowest of any of the subunits of cytochrome c oxidase. The homologies between the bovine and human forms of various subunits are as follows: CIV, 84%; CV, 83%; CVI, 85%; CVII (liver forms), 86%; AED, 86%; STA, 74%. This suggests that subunit CIX has been evolving more rapidly than other subunits of the complex, an idea supported by the observation of polymorphisms in both the heart and liver form of this subunit.

Recently, van Kuilenburg et al. (29) have purified and fully sequenced subunit CIX from human heart. The sequence of this polypeptide is much closer to the sequence of bovine liver than to the bovine heart form (64% cf. 38%) and is identical to the deduced sequence of a cDNA for subunit CIX isolated from a human liver library (25). NaDodSO4-polyacrylamide gels of human heart and liver cytochrome c oxidase show no differences in migration of CIX in contrast to results for beef (Fig. 5), pig, or rat (3). Therefore it is possible that human heart does not contain an H form of CIX.

Both forms of CIX in beef, the heart and liver form, are made as precursors with an N-terminal leader sequence that is removed for assembly of the cytochrome c oxidase complex. Leader sequences have been shown to target polypeptides to the mitochondrion and are thought to be involved in locating polypeptides to their correct compartment, i.e. matrix space, intracristal space, inner membrane or outer membrane (8). Our data on subunit CIX offer the first opportunity to compare two leader sequences which unequivocally contain the same information, both forms of the subunit presumably being incorporated into cytochrome c oxidase in the same specific orientation in the mitochondrial inner membrane. Fig. 3C shows that the homology between the H and L forms of the leader sequences is only 40%, supporting previous claims for the considerable redundancy of the mitochondrial targeting information (8,30). Surprisingly, then, the homology between the leader sequences of bovine liver and human CIX (92%) is higher than that of the mature sequences. The significance of this result is not clear at present.

The L form of CIX has an Arg and the H form a Lys at position -3 from the cleavage site. This is typical of many mitochondrial leader sequences (30). A subset of precursor proteins undergo two cleavage steps to generate the mature polypeptide. Hendrick et al. (30) have shown that such polypeptides are characterized by an Arg at position -10, a hydrophobic residue at -8, and a Ser, Thr, or Gly at -5 from the N terminus of the mature protein. Neither the L or H forms of CIX have this feature and this subunit is probably cleaved to yield the mature polypeptide in a single step. It is important to note that the 5'-untranslated regions of the cDNAs of CIX isolated in this study do not include in-frame stop codons. This is also true of the sequence data reported by Rizzuto et al. (25). Therefore, the possibility that the presequences are longer than shown in Fig. 3 cannot be unequivocally ruled out.
The data from Northern analysis combined with protein chemical studies of cytochrome c oxidase from different tissues give interesting clues about the regulation of isoforms of Crx. In non-muscle tissue, e.g. brain, kidney, and liver, there is transcription of only the L form of Crx, and this is the only isoform found in the isolated complex. In muscle tissue both the H and L isoform mRNAs are present. However, purified cytochrome c oxidase from heart and skeletal muscle contains only the H form as judged by NaDodSO4-polyacrylamide gel electrophoresis (Fig. 5) and based on N-terminal sequencing data. Thus the presence or absence of the H form appears to be determined at the level of transcription while the L form is under posttranscriptional control in muscle tissue. Such post-transcriptional regulation could occur through inhibition of translation of the mRNA for the L form or by a rapid degradation of the translation product as it is synthesized. Alternatively, there could be tissue-specific uptake of isoforms of subunits into the mitochondrion, given the low conservation of the leader sequences of the two isoforms but the very similar leader sequences of the L form in different species.

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