Articles

Sequence of the Bovine 2-Oxoglutarate/Malate Carrier Protein: Structural Relationship to Other Mitochondrial Transport Proteins[†,⊥]

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Received June 15, 1990; Revised Manuscript Received August 22, 1990

ABSTRACT: The amino acid sequence of the 2-oxoglutarate/malate carrier protein, a component of the inner membranes of mitochondria, has been deduced from the sequences of overlapping cDNA clones. These clones were generated in polymerase chain reactions using, in the first instance, complex mixtures of oligonucleotides as primers and probes, with sequences based upon partial protein sequences of cyanogen bromide fragments of the purified protein. The protein sequence of the carrier, including the initiator methionine, is 314 amino acids long. The mature protein has a modified α-amino group, but the nature of this modification and the precise position of the mature N-terminal amino acid have not been ascertained, although it must lie in amino acids 1–4 of the deduced protein sequence. Comparison of the protein sequence with itself and with those of 3 other mitochondrial carrier proteins, ADP/ATP translocase, the phosphate carrier, and the uncoupling protein from brown fat, shows that all 4 proteins contain a 3-fold repeated sequence about 100 amino acids in length, and all the repeats are interrelated. This suggests that the members of this family of proteins have similar structures and mechanisms and that they have evolved from a common origin. The distribution of hydrophobic amino acids in the oxoglutarate/malate carrier supports the view that the domains are folded into similar structural motifs, possibly consisting of two transmembrane α-helices joined by an extensive extramembranous hydrophilic region. Clones of cDNA arising from a longer related transcript of the oxoglutarate/malate carrier gene have also been analyzed. They contain 271 additional nucleotides in the 3′ noncoding region. The same sequence is also present in bovine genomic DNA, and it appears to be an intron. Southern blotting experiments suggest that both the human and the bovine genomes contain single genes for the oxoglutarate/malate carrier and no evidence has been found of different related isoforms in various bovine tissues.

A number of key metabolites involved in oxidative phosphorylation and other important metabolic functions are transported across the inner membranes of mitochondria by proteins [reviewed by La Noue and Schoolwerth (1979) and Kramer and Palmieri (1989)]. The most widely studied of these are the adenine nucleotide transporter, which removes ATP from the matrix in exchange for ADP, and the phosphate carrier. Another mitochondrial transport protein, the oxoglutarate/malate carrier protein, has been isolated from pig and bovine heart mitochondria (Bisaccia et al., 1985; Indiveri et al., 1987). It catalyzes the transport of 2-oxoglutarate across the inner mitochondrial membrane in an electroneutral exchange for malate or other dicarboxylic acids (Palmieri et al., 1972) and plays an important role in several metabolic processes, including the malate–aspartate shuttle, a cyclic pathway to supply reducing equivalents to the electron transport chain (Meijer & van Dam, 1974, 1981). NADH cannot penetrate the mitochondrial inner membrane either by diffusion or by way of a carrier, and, instead, reducing equivalents are transferred indirectly from cytoplasmic NADH to reduce NAD+ in the mitochondrial matrix, where electrons are transferred to NAD+ by malate dehydrogenase. This exchange involves the passage of glutamate and malate into the matrix and 2-oxoglutarate and aspartate in the opposite directions, and requires the participation of both the oxoglutarate/malate and aspartate/glutamate carriers. The oxoglutarate/malate carrier also participates in the oxoglutarate/isocitrate shuttle, in gluconeogenesis from lactate, and in nitrogen metabolism.

Sequence studies of the adenine nucleotide (Aquila et al., 1982; Powell et al., 1989) and phosphate transporters (Runswick et al., 1987; Aquila et al., 1987) have shown that they have related primary stuctures. Their polypeptide chains contain 297 and 313 amino acids, respectively, and each is made of 3 related segments about 100 amino acids in length. All of these repetitive elements could be folded into a similar structural motif consisting of two transmembrane α-helices joined by an extensive hydrophilic extramembrane loop (Saraste & Walker, 1982; Runswick et al., 1987). A third protein, the uncoupling protein found in brown fat mitochondria, also displays similar features and belongs to this family (Aquila et al., 1985). The studies described below identify the oxoglutarate/malate carrier as a fourth member of the family. Its sequence of 314 amino acids deduced from cDNA sequences contains 3 repeats related to those in the known sequences of the other carriers. This suggests that the

[†] This work was supported in part by the target project Ingegneria genetica of CR.

[⊥] The nucleic acid sequence in this paper has been submitted to GenBank under Accession Number J05296.

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[∥] Supported in part by short-term EMBO fellowship.

0006-2960/90/0429-11033$02.50/0

Table I: Synthetic Oligonuleotides Related to the Oxoglutarate/Malate Carrier Protein That Were Employed as Primers or Probes in Polymerase Chain Reactions with cDNA Templates

oligonucleotide[a]	protein sequence used in design[b]	sequence	complexity
1F	MGATVF	ATGGGNGCNACNGTNTT	256
1R	LVKNRM	CATNCKRTTYTTNACNA	512
1P	VQPLDL	GTNCARCCNYTNGAYYT	1024
2F	MISGLV	ATGATHWSNGGNYTNGT	1536
2R	TRIQNM	CATRTTYTGDATNCKNGT	384
2P	MPVDIV	ATGCCNGTNGAYATHGT	96
3F		CTGCTGCCTCCATGCCCGTGGACAT	1
3R	LRDAAP	GGNGCNGCRTCNCKNA	1024
3P	TRIQNM	ACNMGNATHCARAAYATG	384
4F		TTTGTGCAGCCCTTGGACCT	1
4RA		CACATCCAGCCCATTCTTGTATTCT	1
4RB		GGCTTCCCGTCAATCATCCGCATGT	1
4P		CTGCCTCCATGCCCGTG	1
5FA		GCCTGGGCCCCCACACTGTCCTCAC	1
5FB		TTTCATCTTCTTGGAGCAGATGAAC	1
5R		A_{34}	1
5P		GGCCTACAAGCGTCTCT	1
6F		A_{17}	1
6R		AGGTCCAAGGGCTGCACAAA	1
6P	MGATVF	ATGGGNGCNACNGTNTT	256

[a] F and R denote forward and reverse primers, respectively; P denotes oligonucleotide probes. [b] Unique oligonucleotides were based upon sequences of partial cDNA clones characterized from polymerase chain reactions.

four carriers have related structures and mechanisms and that they have evolved from a common ancestor.

Materials and Methods

Isolation of the 2-Oxoglutarate/Malate Carrier. The protein was isolated from bovine heart mitochondria, reconstituted into liposomes, and assayed for transport activity as described by Bisaccia et al. (1985, 1988).

Fragmentation and Sequence Analysis of the Carrier. The N-terminal sequence of a sample of the purified carrier protein was examined in an Applied Biosystems 477 pulsed liquid protein sequencer. A second sample was run on a 10–20% polyacrylamide gradient gel in the presence of sodium dodecyl sulfate and then transferred to a poly(vinylidene difluoride) (pvdf) membrane (Matsudaira, 1987; Fearnley et al., 1989). The single band detected by staining with Coomassie blue dye was excised and subjected to Edman degradation in a modified Applied Biosystems 470 gas-phase protein sequencer (Fearnley et al., 1989). Another sample of the carrier protein was dissolved in 70% formic acid and digested under argon for 4 h with cyanogen bromide. The products were fractionated by reverse-phase HPLC on a C_{18} Nucleosil column in 0.1% trifluoroacetic acid with a linear gadient of acetonitrile, and each peak detected at 225 nm was examined by sequence analysis in the gas-phase instrument. Another sample (100 μg) was digested with porcine elastase (2 μg) for 18 h at 18 °C in a buffer (200 μL) containing Tris·HCl, pH 8.8, and sodium dodecyl sulfate (0.1%). Then the digest was boiled for 2 min, and the products were fractionated by polyacrylamide electrophoresis on a 16.5% polyacrylamide gel in the presence of 6 M urea (Schägger & von Jagow, 1987), transferred by electrophoresis to a pvdf membrane, detected and excised as described above, and analyzed in a gas-phase protein sequencer.

Oligonucleotide Synthesis. Syntheses were performed in an Applied Biosystems 320B DNA synthesizer. Some of the oligonucleotides were used as primers in polymerase chain reactions (see Table I and below) and others as hybridization probes to investigate the products of these reactions. Forward and reverse primers were made with *Eco*RI or *Hin*dIII linkers, respectively, on their 5′ ends. Some unique synthetic oligonucleotides 17 bases long were employed as primers in sequencing reactions. In all cases, synthetic oligonucleotides were purified by absorption on a C_{18} Sep-Pak column (Waters Associates, Milford, MA) followed by elution with 60% methanol. Conditions for radiolabeling oligonucleotides and for their use as hybridization probes have been described before [see Gay and Walker (1985a), Powell et al. (1989), and Walker et al. (1989)].

Polymerase Chain Reactions. Amplifications of segments of cDNA or genomic DNA by means of the polymerase chain reaction were carried out by using a Techne programmable Dri-Block, Model PHC-1. Single-stranded and double-stranded cDNAs were made from poly(A+) mRNA (see below for details of its preparation) using a cDNA synthesis kit (Amersham International, Amersham, U.K.). The single-stranded cDNA (derived from 1.5 μg of mRNA) was tailed with a run of A residues at its 5′ end in a buffer (15 μL) containing 100 mM potassium cacodylate, pH 6.9, 0.1 mM dithiothreitol, 1 mM $CoCl_2$, 1 mM dATP, bovine serum albumin (0.5 μg), and terminal deoxynucleotide transferase (36 units; Pharmacia, Milton Keynes, U.K.). The reaction was carried out at 37 °C for 30 min. The following schedule was used in polymerase chain reactions: denaturation at 94 °C for 2.0 min (later reduced to 1 min); annealing for 2.0 min either at 5 °C below the minimum dissociation temperature (Suggs et al., 1981) or at 40 °C, whichever was the greater; synthesis for 2 min at 70 °C for 35 cycles; and finally a single incubation at 70 °C for 7 min. The reactions were performed in a solution (100 μL) containing 50 mM potassium chloride, 10 mM Tris-HCl, pH 8.3, 1.5 mM magnesium chloride, each dNTP (200 μM) both primers (2 μM), cDNA (10 ng), and *Thermus aquaticus* DNA polymerase (3 units; Perkin Elmer Cetus Corp., Norwalk, CT). The reaction mixture was prepared without enzyme and incubated at 94 °C for 2.5 min. Then the enzyme was added and the reaction mixture covered with mineral oil (100 μL; Sigma Chemical Co. Ltd., Poole, Dorset, U.K.). In experiments, PCR4 and PCR5 pairs of nested primers (primers 4RA and 4RB, 5FA and 5FB, respectively) in the same sense of the DNA were used with a single primer in each case in the opposite sense (primers 4F and 5R, respectively; see Table I). In each experiment, the first of the nested pair (4RA or 5FA) was added at a concentration of 25 nM, and then 35 cycles of synthesis were conducted in the presence of the reverse primers (4F or 5R). Then the second member of the nested pair (4RB or 5FB) was

added at a concentration of 1 μM, and a further 35 cycles of synthesis were performed. The products of polymerase chain reactions were analyzed on 1.4% high-melting agarose gels in a buffer containing 90 mM Tris, 90 mM boric acid, 2.5 mM EDTA, and ethidium bromide (1 μg/mL). DNA fragments were transferred to Hybond-N membranes (Amersham International), cross-linked to the membrane by irradiation with UV light, and hybridized with radioactively labeled synthetic oligonucleotides at 5 °C below the minimum dissociation temperature. Fragments that hybridized with the probe were recovered from the gel, the *Hin*dIII and *Eco*RI sites in the linker sequences were hydrolyzed, and the products were cloned into M13 mp18 and mp19 vectors that had been treated with the same restriction enzymes. One experiment was performed to amplify a region of bovine genomic DNA containing part of the gene for the oxoglutarate/malate carrier. Two pairs of nested primers were employed. The first pair corresponded in the cDNA sequence to nucleotides 895–919 (forward) and 1133–1157 (reverse), and the second pair was nucleotides 920–944 (forward) and 1108–1132 (reverse). In addition, these latter oligonucleotides were synthesized with *Eco*RI and *Hin*dIII linkers on forward and reverse primers, respectively. About 1 μg of genomic DNA was used, with final primer concentrations of 25 nM and 1 μM for the first and second pairs, respectively. The polymerase chain reaction schedule was as follows: denaturation at 94 °C for 1 min; annealing for 2 min at 55 °C; synthesis for 2 min at 72 °C for 30 cycles with each pair of primers; and finally a single incubation at 72 °C for 7 min. Otherwise, the reactions and the analysis of products were carried out as described above for cDNA.

Hybridization with Genomic DNA and with RNA from Various Tissues. Bovine and human genomic DNAs were prepared from liver and placenta, respectively, as described previously (Walker et al., 1987a). Total RNA was extracted from bovine heart, liver, and brain by the method of Chirgwin et al. (1979), and poly(A+) RNA was purified from total RNA as described before (Viñas et al., 1990). Samples of genomic DNA (10 μg) were digested with one of the restriction endonucleases, *Eco*RI, *Hin*dIII, *Kpn*I, and *Sac*I, and the digests were fractionated in a 0.6% agarose gel. The DNA fragments were transferred to Hybond N membranes by the method of Southern (1975) and were hybridized with radioactively labeled "prime-cut" probe consisting of nucleotides 159–817 of the compiled cDNA sequence (Farrell et al., 1983). The same probe was also employed in Northern hybridizations with samples of mRNA prepared from various bovine tissues. Hybridizations with both genomic DNA and mRNA were performed at 65 °C in a solution containing 6 × SSC (SSC is 0.15 M NaCl/0.015 M sodium citrate), 5 × Denhardt's solution [which contains 1 mg/mL each of poly(vinylpyrrolidone), bovine serum albumin (fraction V), and ficoll], boiled sonicated salmon sperm DNA (1 mg/mL), and 0.1% sodium dodecyl sulfate. Other experimental procedures concerning hybridization have been described before (Walker et al., 1987b; Viñas et al., 1990).

DNA Sequence Analysis. DNA sequences were determined by the modified dideoxy chain termination method (Sanger et al., 1977; Biggin et al., 1983). Data were compiled and analyzed with the computer program DBUTIL (Staden, 1982a) and ANALYSEQ (Staden, 1985).

Results and Discussion

Partial Protein Sequence Analysis of the Oxoglutarate/Malate Carrier Protein. No N-terminal sequence was detected when samples of the intact carrier protein were subjected to the Edman degradation. So it appears that the protein has a modified α-amino group, although the nature of this modification has not been ascertained. Partial internal protein sequences were obtained from fragments isolated from cyanogen bromide and elastase digests of the carrier. The results of these experiments are summarized in Table II. With one exception, all of these sequences were eventually found to originate from the oxoglutarate/malate carrier. The exception is the final sequence listed in Table II, which at first was interpreted as arising from a single peptide, but this interpretation was changed later (see below).

Table II: Protein Sequences Determined on the 2-Oxoglutarate Carrier Protein and on Digestion Products

sample	determined sequence[a]	position in protein
intact protein	none	
elastase digest	ASPGASGMDGKP...	5–16
cyanogen bromide	DGKPRTS...	13–19
cyanogen bromide	GATVFVFQPLDLVKARHse	34–49
cyanogen bromide	ARAVVVNAAQLASYSQSKQFLL-	189–210
cyanogen bromide	ISGLVTTAASMPVDIVKTRIQNHse	228–250
cyanogen bromide	ISGLVTTAASMPVDIVKTRIQNMRHse	228–252
cyanogen bromide	NKAYKRLFLSG[GYANTLRDAAP...][b]	304–314

[a] See Figure 2. [b] The sequence in brackets is not part of the 2-oxoglutarate carrier protein (see text).

Isolation and Sequence Analysis of Complementary DNA Clones Encoding the 2-Oxoglutarate/Malate Carrier. On the basis of the partial protein sequences obtained from cyanogen bromide fragments of the oxoglutarate/malate carrier protein, a number of mixtures of oligonucleotides 17 bases in length were synthesized and employed as hybridization probes in attempts to isolate clones encoding the carrier from a bovine cDNA library (Gay & Walker, 1985b). All of these experiments were unsuccessful. In contrast, similar experiments to isolate clones for a range of other components of the mitochondrial membrane by the same procedure were conducted successfully [summarized in Runswick et al. (1990)].

Therefore, it was decided to adopt a different strategy based upon the polymerase chain reaction. This strategy, which we have since developed and extended and applied successfully to about 20 other proteins, requires only short segments of protein sequence (about 18 amino acids) such as were available in this case. In a first round of polymerase chain reactions, a short cDNA is generted by using forward and reverse primers at the N- and C-terminal extremities of the segment of available sequence, respectively. These primers are 17 bases in length (plus appropriate linker sequences; see Materials and Methods), and primers of this length with complexities from 48 to 4096 have been used successfully in similar experiments (J. E. Walker, A. Dupuis, I. M. Fearnley, M. Finel, S. M. Medd, S. J. Pilkington, M. J. Runswick, and J. M. Skehel, umpublished experiments). The products of the reaction are cloned into an M13 vector, and appropriate clones containing the sequence of interest are recognized with a synthetic hybridization probe based upon the central region of the available protein sequence. These probes again are usually 17 bases long, and their complexities have ranged up to 2048 in successful experiments. By this procedure, a short cDNA encoding the segment of available protein sequence is obtained. However, because mixed primers are used in the polymerase chain reaction, only the central part of the sequence between the two primers is accurate. So, this central sequence is used as the basis of further experiments to generate the complete cDNA. Here a number of routes have been followed. The simplest, and most direct, is to generate a cDNA by the polymerase chain reaction up to the 3′ extremity using part of

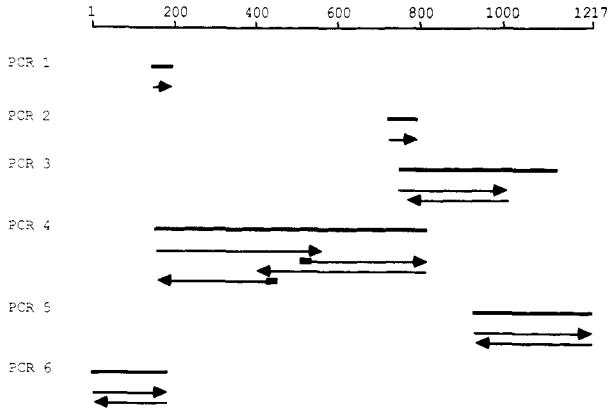

FIGURE 1: Generation by the polymerase chain reaction and sequence analysis of clones encoding the 2-oxoglutarate/malate carrier from bovine heart mitochondria. PCR1–PCR6 are the partial cDNAs generated by polymerase chain reactions (for details, see the text), and the heavy horizontal lines are proportional to the lengths of these cDNAs. The arrows represent the directions and extents of the determined DNA sequences, and the boxes attached to the arrows denote synthetic oligonucleotide primers used in sequencing reactions. The scale is in bases.

the unique sequence as a forward primer, and oligo(dT) as a reverse primer annealed to the 3′ poly(A) sequence. Similarly, a clone up to the 5′ extremity can be produced by using part of the unique sequence as a reverse primer and oligo(dT) as a forward primer on cDNA to which poly(A) has been added to the 5′ end with terminal transferase. In both experiments, the remainder of the unique cDNA sequence is employed as a hybridization probe.

In the case of the oxoglutarate/malate carrier protein, a similar, but somewhat more complex, approach was followed, as outlined in Figure 1. In summary, this shows that two segments of protein sequence were used to generate corresponding short cDNA clones in the manner described above (reactions PCR1 and PCR2 in Figure 1). A third similar experiment (not described) was carried out with the protein sequence NKAYKRLFLSGGYANTLRDAAP (see Table II), but no product that hybridized with the probe was obtained. Still working upon the assumption (which proved to be incorrect) that this protein sequence was present in the oxoglutarate/malate carrier, we performed another polymerase chain reaction experiment (PCR3) inter alia in which the unique forward primer 3F was used with the mixed reverse primer 3R. This generated a product that hybridized with the degenerate probe ACNMGNATHCARAAYATG (based upon the protein sequence TRIQNM). However, sequence analysis showed that the cDNA coded for the protein sequence NKAYKRLFLSG but that this was followed immediately by a stop codon and that a sequence coding for the protein sequence GYANTLRDAAP was absent. This latter sequence is identical with the sequence of amino acids 146–156 of the mitochondrial phosphate carrier protein. Reinspection of the protein sequence data confirmed that the sample from which the protein sequence originated was in reality a mixture of two peptides originating from the C-terminal region of the oxoglutarate/malate carrier and residues 135–156 of the phosphate carrier. In the first part of the sequencing experiment, the peptide from the oxoglutarate/malate carrier was predominant, and that starting from amino acid 135 of the phosphate carrier was minor. As the degradation proceeded, the former sequence diminished rapidly, leaving only that derived from the phosphate carrier after Edman cycle 11. This sequence, now attributed to contaminating phosphate carrier, was the only one detected in three separate cyanogen bromide digests of different preparations of the oxoglutarate/malate carrier that originated from the phosphate carrier, and all other determined sequences are present in the complete sequence of the oxoglutarate/malate carrier (see below).

It is now apparent that the synthesis of the cDNA in polymerase chain reaction PCR3 depended upon the fortuitous hybridization of the mixed reverse primer 3R with the sequence TAGGAGGACGTGGCCC present in the 3′ noncoding region of the cDNA for the oxoglutarate/malate carrier. The DNA sequence through this region has also been determined in bovine genomic DNA and confirms the region coding for the C-terminus of the protein, the stop codon, and the 3′ noncoding region up to the poly(A) tail (see below).

The sequence of the cDNA for the oxoglutarate/malate carrier was completed in three further experiments in which the clones were generated by the polymerase chain reaction. These are experiments PCR4–PCR6 in Figure 1. In experiment PCR4, the unique sequences produced in experiments PCR1 and PCR3 were joined together; then the sequence generated in PCR3 was extended in a 3′ direction by priming from the poly(A) tail (experiment PCR5); finally, in experiment PCR6, the sequence produced in PCR1 was extended in a 5′ direction by priming on poly(A) that had been added to the 5′ end of the total bovine cDNA with terminal transferase (see Materials and Methods).

The complete cDNA sequence, shown in Figure 2, is compiled from the shorter overlapping cDNA clones and has been determined completely in both senses (see Figure 1). It is 1217 nucleotides in length and is terminated by a run of A residues separated by a sequence of 22 nucleotides from the preceding sequence AATAAA, a typical signal for polyadenylation of RNA (Proudfoot & Brownlee, 1976). In experiment PCR5, an additional clone was isolated which differed from other clones covering this region analyzed in the same experiment and also in experiment PCR3, since it contained an additional 271 nucleotides between nucleotides 997 and 998. This extra sequence contains at its 5′ and 3′ extremities, respectively, the dinucleotide sequences GT and AG, which are conserved at the 5′ and 3′ boundaries of introns (Breathnach & Chambon, 1981). Therefore, it seemed likely that this longer clone arose from a partially spliced transcript and that the extra sequence is an intron. In order to demonstrate this arrangement in genomic DNA and to eliminate any possibility that the extra sequence had a spurious origin, a region of bovine genomic DNA was amplified by the polymerase chain reaction using forward and reverse primers taken from nucleotides 895–944 and 1108–1157, respectively (see Materials and Methods for experimental details). If the proposed intron were present, as predicted, this would be expected to give a product 485 nucleotides long. When the products of this reaction were separated by agarose gel electrophoresis, ethidium bromide staining revealed a single band with an estimated size of about 500 nucleotides. Its sequence was identical with that of nucleotides 919–1132 of the cDNA, and the same additional sequence of 271 bases were present between nucleotides 997 and 998. No intron is found at the corresponding positions of the two expressed human genes, T1 and T2, encoding the ADP/ATP translocase (Cozens et al., 1989).

Protein Sequence of the Oxoglutarate/Malate Carrier. The compiled cDNA sequence encodes a protein of 314 amino acids. The initiation codon (nucleotides 48–50) can be identified with some certainty since a potential termination codon, TGA, is found in the same phase of the DNA at nucleotides 39–41. At residues 5–19 of the protein is found a sequence that is identical with a protein sequence determined on a

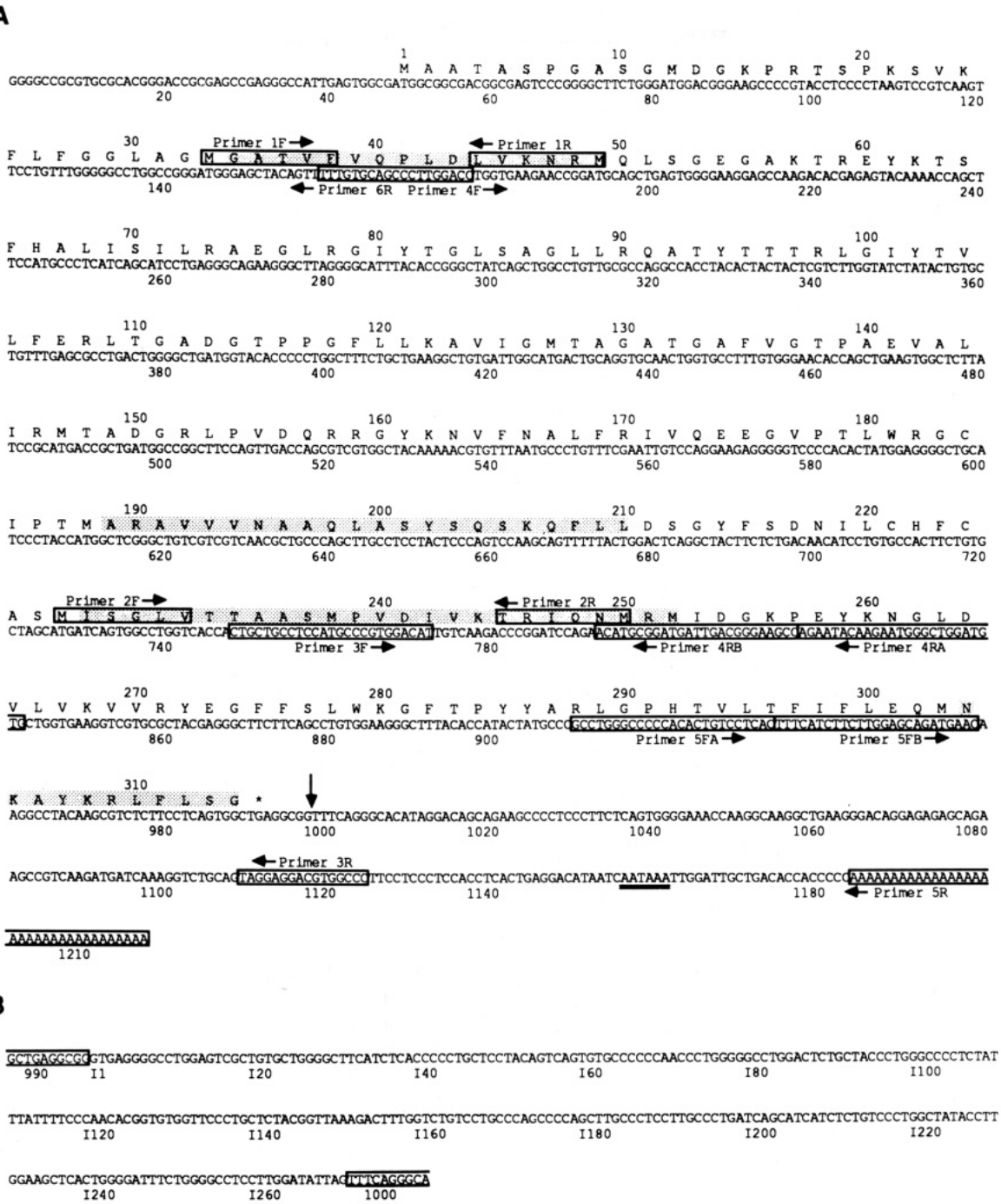

FIGURE 2: Compiled sequence of cDNAs encoding the 2-oxoglutarate/malate carrier from bovine heart mitochondria. In (A), the sequence is shown without an extra sequence, proposed to be an intron, present in one cDNA clone in experiment PCR5. This proposed intron was shown to be present in bovine genomic DNA, and its sequence together with flanking exon sequences is shown in part B. In (A), the deduced protein sequence is numbered from 1 to 314. The precise position of the modified amino-terminal residue is unknown, but must be in one of amino acids 1–4. Protein sequences determined by sequence analysis of fragments isolated from cyanogen bromide and elastase digests of the oxoglutarate/malate carrier protein are shaded. Boxed protein sequences were used to design mixtures of oligonucleotides employed as primers in polymerase chain reactions (see Table II). Boxed nucleotide sequences are those of unique primers, except for that denoted as 3R; this is the site of fortuitous priming of the mixed primer 3R (see text and Table I). Horizontal arrows pointing to the left or right indicate that the primers were synthesized as the sequence shown or its complement, respectively. A vertical arrow is placed in the 3′ noncoding region between nucleotides 997 and 998 at the position of a proposed intron. The underlined sequence AATAAA is a potential polyadenylation signal. In (B), the flanking exon sequences are boxed, and the proposed intron sequence is numbered I1–I271.

fragment isolated from an elastase digest of the mature oxoglutarate/malate carrier (see Table II). Therefore, in common with the ADP/ATP translocase and the brown fat uncoupling protein, but in contrast to the phosphate carrier (Runswick et al., 1987), the oxoglutarate/malate carrier has no processed N-terminal extension to specify its entry into mitochondria, and the import signal must lie within the mature protein. The N-terminus of the mature protein is modified in an unknown way after translation, and the initial translation product may also have undergone some posttranslational proteolytic processing in vivo. However, because residues five onward have been shown to be present in the mature protein, if such processing happens in vivo it must be limited and cannot extend beyond residue 4. Also, since elastase has no known protein deacylase activity, the modified N-terminal residue must be in the first four amino acids. The molecular weight calculated

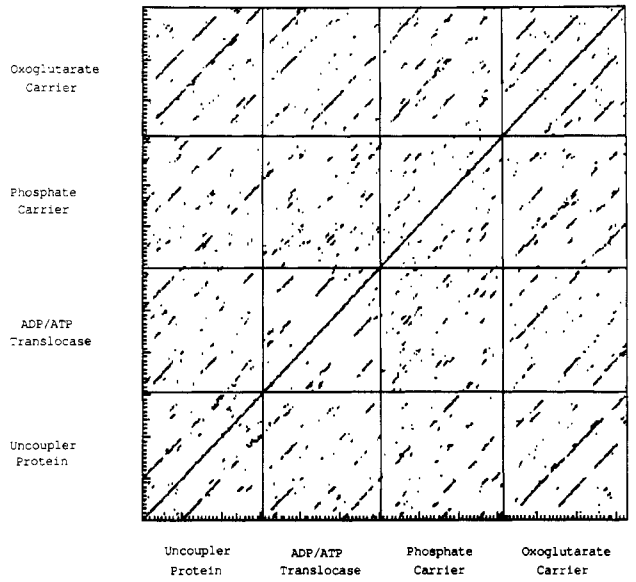

FIGURE 3: Binary comparisons of sequences of mitochondrial carrier proteins. The calculation was made with the computer program DIAGON (Staden, 1982b) with a window of 25 amino acids and a score of 275.

from the sequence of the unmodified protein is 34 172, and is in reasonable agreement with the value of 31 500 estimated for the pig and cow proteins by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Bisaccia et al., 1985; Indiveri et al., 1987).

Repetitive Sequences in Mitochondrial Carrier Proteins. Comparison of the sequence of the oxoglutarate/malate carrier protein with itself reveals that, as in the cases of the ADP/ATP translocase, the phosphate carrier, and the brown fat uncoupling protein, it has a tripartite structure made of related sequences about 100 amino acids in length (see Figure 3). Moreover, the repetitive elements are related to those found in the other carriers. Therefore, the four proteins have evolved from a common ancestor and have related structures and mechanisms. The structural relationship between the four proteins is further emphasized by the similarities in their hydrophobic profiles (see Figure 4). These profiles have been discussed previously in relation to the ADP/ATP (Saraste & Walker, 1982) and phosphate carriers (Runswick et al., 1987) and also for the uncoupling protein (Aquila et al., 1985). They can be interpreted as indicating that the carriers contain six hydrophobic domains capable of being folded into membrane-spanning α-helices, and the profile of the oxoglutarate/malate carrier is consistent with this proposition. These potential membrane-spanning segments are indicated by I–VI in Figure 4. According to this model, in each repetitive domain, the two helices are linked by an extensive extramembranous loop (A, B, and C in Figure 4). Alignment of the 12 related sequences on the basis of the sequence comparisons (see Figure 5) indicates that the proposed extramembranous regions, which are hydrophilic, are among the most variable parts of the proteins and they differ extensively in length. In contrast, the junctions between the hydrophobic and hydrophilic regions are the most highly conserved, and presumably they play key roles in the structure and function of the carriers, while carrier specificity is conferred by some of the poorly conserved residues. This model for the carriers, based upon the tripartite repeated sequence and the hydrophobic profiles, is by no means the only one that could be proposed. However, the tripartite places restraints on possible models since it strongly implies that each of the three domains in the four proteins will have related secondary and tertiary

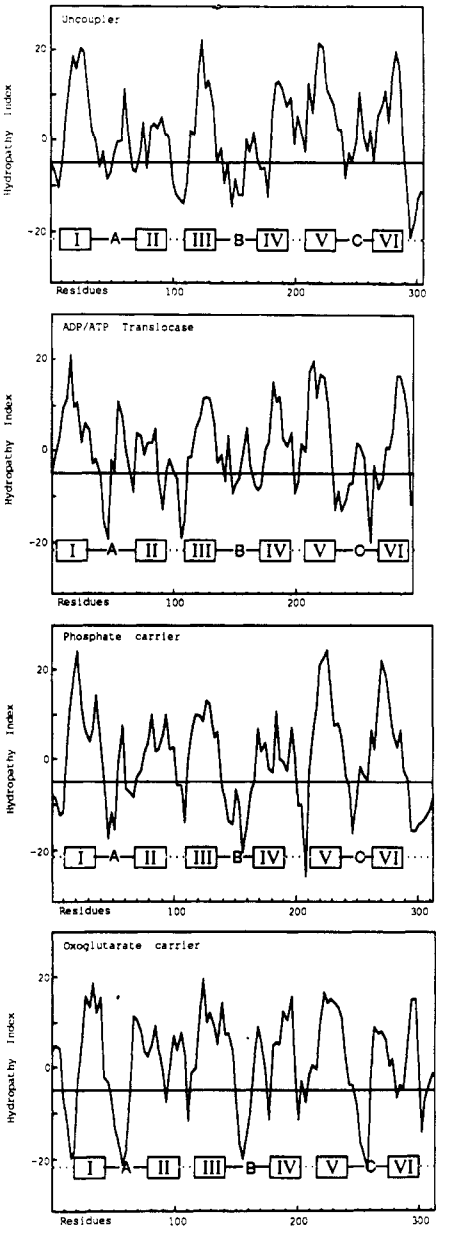

FIGURE 4: Comparison of hydrophobic profiles of mitochondrial carrier proteins. The calculations were made with the computer program HYDROPLOT, a version of SOAP (Kyte & Doolittle, 1982).

structures, and therefore models that propose different secondary structures for the first and second domains, for example (Aquila et al. (1987), are less likely to be correct. It is noticeable that in the ADP/ATP carrier the hydrophobicities of segments I, III, and V are greater than those of segments II and IV, but not VI, and the same is true in the cases of the phosphate carrier and the uncoupling protein. This could be interpreted as suggesting that these less hydrophobic regions do not span the bilayer in α-helices. However, the hydrophobicity of segment II in the oxoglutarate/malate carrier is enhanced. At present, there is no decisive experimental evidence to support or disprove these various models.

The only available experimental data concerning the topology of the oxoglutarate/malate carrier concern its cysteine residues. It has been demonstrated in mitochondria, but not in submitochondrial membranes (everted inner mitochondrial membranes), that the bovine carrier is inhibited by eosinyl-5-maleimide (Zara & Palmieri, 1988). Therefore, the modified thiol group(s) is (are) exposed from the outer surface of the inner membrane and not from the inner surface. The three

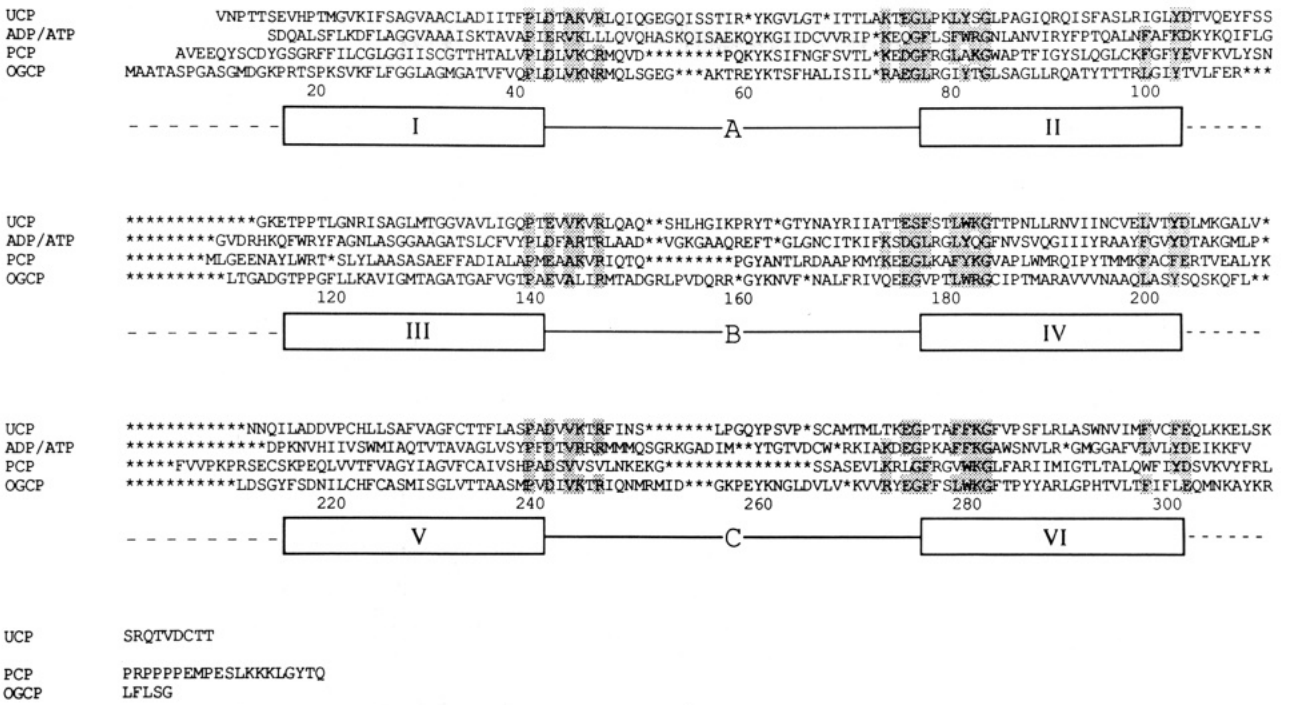

FIGURE 5: Alignment of repetitive sequence elements in mitochondrial carrier proteins. The alignments are based upon the sequence comparisons shown in Figure 3. Amino acid residues that are conserved in 8 out of the 12 sequences are shaded. I–VI represent hydrophobic regions (see Figure 4) that may be folded into α-helices, and A, B, and C are hydrophilic segments that are proposed to lie outside the lipid bilayer.

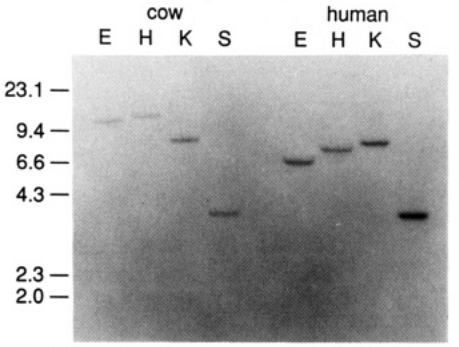

FIGURE 6: Hybridization of restriction digests of bovine DNA with probes derived from the cDNA for the bovine heart 2-oxoglutarate/malate carrier. The DNA was digested with the restriction enzymes *Eco*RI, *Hin*dIII, *Kpn*I, and *Sac*I, denoted by E, H, K, and S, respectively. The probe was nucleotides 159–817 (see Figure 2 and Materials and Methods for experimental details).

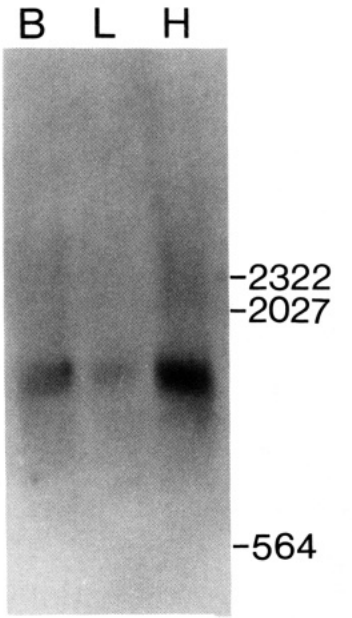

FIGURE 7: Hybridization of poly(A+) RNA from bovine tissues with probes derived from the cDNA for the 2-oxoglutarate/malate carrier from bovine heart mitochondria. The tissues brain, heart, and liver are denoted by B, H, and L, respectively. The same hybridization probe was employed as in Southern blots on genomic DNA (see legend to Figure 6).

cysteine residues in the sequence of the oxoglutarate/malate carrier protein (residues 184, 221, and 224) all lie within relatively hydrophobic regions of the protein that could be folded into membrane-spanning α-helices (see below).

Number of Genes for the Oxoglutarate/Malate Carrier. A number of mammalian mitochondrial membrane proteins have more than one expressed gene that codes for isoforms. Perhaps the most notable in this respect is the ADP/ATP translocase. Two different cDNAs, named T1 and T2, have been characterized and encode homologues of the translocase that differ in 33 amino acids out of 297 (Walker et al., 1987c; Powell et al., 1989). They have been shown to be expressed in widely different ratios in various bovine tissues; T1 is expressed predominantly in heart, whereas T2 is found mainly in intestine (Powell et al., 1989). Human cDNAs for T1 and T2 were characterized subsequently (Neckleman et al., 1987; Houldsworth et al., 1988), and the human T1 and T2 genes have been sequenced (Cozens et al., 1989). In addition, a third human homologue, T3, is expressed in HL60 cells (Battini et al., 1987). In contrast, only one form of the phosphate carrier has been detected (Runswick et al., 1987). In the case of the oxoglutarate/malate carrier, antibodies have been raised to the bovine heart and the rat liver proteins (Zara et al., 1990). The cow anti-heart antiserum cross-reacts with the heart proteins in pig, rat, and rabbit, but not with the liver proteins in the same species or in cows. Conversely, the antiliver antiserum recognizes the liver but not the heart proteins in the same animals. Therefore, we have sought for additional evidence for isoforms of the oxoglutarate/malate carrier by using the bovine cDNA as a hybridization probe. By Southern blot

analysis of digests of bovine and human DNA, single hybridizing bands have been detected (see Figure 6), and by Northern blot analysis, hybridizing transcripts were found in bovine heart, liver, and brain (see Figure 7). These experiments, and also polymerase chain reactions performed on bovine genomic DNA templates (described above), suggest that the bovine genome contains a single gene for the oxoglutarate/malate carrier and that there are no isoforms with closely related gene sequences. They also show that the single isoform that has been detected is expressed at least in heart, liver, and brain tissues. However, they do not exclude the possibility of the existence of other more distantly related sequences coding for isoforms of this protein, and the immunological experiments can be explained by the existence of such a distant relative. An alternative explanation is that the heart and liver forms detected by the antibodies are products of the same gene that have been modified differently after translation.

ACKNOWLEDGMENTS

We are grateful to Mr. T. V. Smith and Mrs. J. Fogg for their help in the synthesis of oligonucleotides.

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