Apolipoprotein J: Structure and Tissue Distribution†,‡

Harshini V. de Silva, Judith A. K. Harmony,* William D. Stuart, Cleris M. Gil, and Jeffrey Robbins
Division of Lipoprotein Research, Department of Pharmacology and Cell Biophysics, University of Cincinnati College of Medicine, 231 Bethesda Avenue, Cincinnati, Ohio 45267-0575

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ABSTRACT: The primary structure of apolipoprotein J (apoJ) was deduced by the combined strategies of protein sequencing and cDNA cloning and sequencing. ApoJ, an apolipoprotein associated with discrete subclasses of high-density lipoproteins, is encoded by a single gene in both the human and mouse genomes. ApoJ is synthesized as a 427 amino acid polypeptide that is posttranslationally cleaved at an internal bond between Arg-205 and Ser-206. The subunits of apoJ are designated apoJα, corresponding to residues 1–205, and apoJβ, corresponding to residues 206–427. The subunits are associated through disulfide bonds. Analysis of the primary structure of apoJ predicts the existence of amphiphilic helices, which may account for the association of apoJ with lipoproteins, and heparin-binding motifs in both subunits. ApoJ appears to be the human analogue of a rat protein present in high concentrations in the testis, sulfated glycoprotein 2. ApoJ mRNA (1.9 kb) is expressed in all but one tissue examined. The mRNA is present in relatively high levels in brain, ovary, testis, and liver, is less abundant in heart, spleen, lung, and breast, and is absent in T-lymphocytes. ApoJ is unique among previously characterized human apolipoproteins in its structure and tissue distribution.

Apolipoprotein J (apoJ) is a 70-kDa protein associated with high-density lipoproteins (HDL)[1] in human plasma (de Silva et al., 1990a). HDL and low-density lipoproteins (LDL) transport >80% of the plasma cholesterol in humans. Recently, attention has focused on HDL since the amount of HDL–cholesterol is inversely correlated with the risk of premature atherosclerosis (Castelli et al., 1977). The antiatherogenic nature of HDL is attributed, in part, to its potential role in the transport of cholesterol from peripheral tissues—which are unable to catabolize excess cholesterol—to the liver for clearance, a process referred to as reverse cholesterol transport (Glomset, 1968). Surprisingly little is known about the origin or metabolic fate of HDL. A complicating factor is the number of HDL subclasses (Kane, 1986; Krauss & Nichols, 1986). Using immunoaffinity chromatography, we recently identified discrete subclasses of HDL, each of which consists of apoJ and apoAI (de Silva et al., 1990a). The apoJ–apoAI subclasses can distribute with HDL_2 (d = 1.063–1.125 g/mL) and HDL_3 (d = 1.125–1.21 g/mL) but are most abundant in the class of very high density lipoproteins (VHDL, d = 1.21–1.25 g/mL). ApoJ can serve as a marker of discrete subpopulations of HDL and may be used to further our understanding of the metabolism of HDL in normal situations and disease states.

ApoJ has been purified from human plasma by a two-step procedure of immunoaffinity chromatography and reverse-phase high-performance liquid chromatography (RP-HPLC) and characterized (de Silva et al., 1990b). ApoJ is comprised of two disulfide-linked subunits designated apoJα (34–36 kDa) and apoJβ (36–39 kDa), each of which has a pI of 4.9–5.4. When chemically deglycosylated, the subunits have molecular masses of 24 and 28 kDa, respectively. A major problem unsolved in previous investigations is the structural relationship between apoJα and apoJβ. At least partial homology between apoJα and apoJβ is indicated by their similar molecular weights, amino acid compositions, and isoelectric points. Moreover, two of six monoclonal antibodies (mAb) specific for apoJ, and which recognize protein epitopes, react with both apoJα and apoJβ. However, other lines of evidence suggest very little, if any, similarity between apoJα and apoJβ. Four apoJ mAb react with apoJα only. The amino-terminal 30 residues of apoJα and apoJβ are distinct, and peptide maps of each subunit, constructed following tryptic or *Staphylococcus aureus* V8 protease digestion, reveal few similarities. The molecular weights of the primary translation products, immunoprecipitated with individual apoJ mAb following translation of HepG2 mRNA in vitro, are the same regardless of whether the mAb recognize only apoJα or both apoJα and apoJβ. The simplest model which integrates all of these results (de Silva et al., 1990b) is that apoJα and apoJβ are derived from a common protein precursor.

This investigation was initiated to define further the structure of apoJ and to establish the relationship between the α and β units of apoJ. The strategy involved a combination of protein sequencing and cDNA cloning. We reasoned that, once the primary structure of apoJ was known, these data together with information about the tissue distribution of the apoJ message might be used to formulate testable hypotheses concerning the role of apoJ in HDL metabolism. We have determined the primary structures of apoJα and apoJβ and show that they are encoded by the same gene, being synthesized in tandem as a primary translation product of 50 kDa.

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*Address correspondence to this author.

[1] Abbreviations: apo, apolipoprotein; BSA, bovine serum albumin; DBM, [(diazobenzyl)oxy]methyl; HDL, high-density lipoproteins; LDL, low-density lipoproteins; mAb, monoclonal antibodies; RP-HPLC, reverse-phase high-performance liquid chromatography; PFU, plaque forming units; PVP, poly(vinylpyrrolidone); SDS, sodium dodecyl sulfate; SSC, standard saline citrate containing 150 mM sodium chloride and 15 mM sodium citrate; SGP2, sulfated glycoprotein 2; VHDL, very high density lipoproteins.

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ApoJα and apoJβ are produced by posttranslational proteolysis at an internal Arg–Ser bond. The tissue distribution of apoJ mRNA is similar to that of apoE (Driscoll & Getz, 1984; Elshourbagy et al., 1985; Lin-Lee et al., 1985; Williams et al., 1985; Zannis et al., 1985).

Materials and Methods

Amino Acid Sequencing. The apoJ subunits were purified by RP-HPLC and digested with trypsin or *S. aureus* V8 protease (de Silva et al., 1990b). The major peptides from each digest were initially separated by RP-HPLC, with 0.1% trifluoroacetic acid and a gradient of 0–90% acetonitrile, and rechromatographed, with 4.5 mM heptafluorobutyric acid as an ion pairing agent and a gradient of 0–90% acetonitrile. The purified peptides were sequenced with an Applied Biosystems gas-phase sequencer; phenylthiohydantoin-derivatized amino acids were analyzed with a Waters HPLC, using a NOVA-PAK C_{18} column. Amino acid sequence was deduced from the cDNA, and secondary structure predictions and nucleic acid and protein bank searches were carried out with DNANALYZE (Wernke & Thompson, 1989).

Isolation of RNA. The human hepatocarcinoma cell line HepG2 (Knowles et al., 1980) was purchased from the American Type Culture Collection. The cells were grown to confluence in minimal essential medium (MEM), containing Earles' salts supplemented with 10% fetal bovine serum (FBS) and 4 mM L-glutamine. Total RNA from HepG2 cells was isolated as described (Chirgwin et al., 1979) with modifications (de Silva et al., 1990b). Messenger RNA (mRNA) was purified by oligo(dT)–cellulose (type 7, Pharmacia LKB Biotechnology) chromatography (Aviv & Leder, 1972). Human blood T-lymphocytes were provided by Michael Kelly (Developmental Biology Program, University of Cincinnati, Cincinnati, OH). Total RNA from human tissues was isolated by the RNAzol method adapted from Chomczynski and Sacchi (1987), according to the manufacturer's instructions (Cinna/Biotecx Labs). Integrity of RNA preparations was assessed by methylmercury–agarose gel electrophoresis (Bailey & Davidson, 1976).

Synthesis and Labeling of DNA Probes. Oligonucleotide probes were synthesized on the basis of the amino-terminal sequences of apoJα and apoJβ. The oligonucleotides (each 71 bases in length), designed with preferred codons (Lathe, 1985) and deoxyinosines (Ohtsuka et al., 1985) in the wobble position, were synthesized by the Molecular Biology Core Facility, Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati. Oligonucleotides were purified on 12% polyacrylamide gels by electrophoresis (Maxam & Gilbert, 1977). The oligonucleotides were end labeled (Maniatis et al., 1978) with [γ-^{32}P]ATP (~6000 Ci/mmol; NEN Du Pont Co.), by use of polynucleotide kinase (International Biotechnologies Inc.).

Hybridization of Oligonucleotides with RNA. RNA, electrophoresed through methylmercury gels, was transferred overnight at 4 °C to activated [(diazobenzyl)oxy]methyl (DBM) paper (Schleicher & Schuell Inc.) (Alwine et al., 1977) in 0.2 M sodium acetate, pH 4.2. Unoccupied sites were blocked at 60 °C for 1 h with 2.5× standard saline citrate (SSC), pH 6.5, containing 1% glycine and 0.1% sodium dodecyl sulfate (SDS). Prior to hybridization with the oligonucleotides, the blotted RNA was incubated at 42 °C for 1 h in 6× SSC, 1% SDS, 0.1% poly(vinylpyrrolidone) 40 (PVP), 0.1% bovine serum albumin (BSA, fraction V), 0.1% Ficoll (type 400), and 100 μg/mL denatured salmon sperm DNA. Hybridization with the cDNA probes was performed at 60 °C in 4× SSC, 0.1% SDS, 0.1% PVP, 0.1% BSA, 0.1% Ficoll, and 100 μg/mL salmon sperm DNA. The blots were hybridized for 12 h with end-labeled oligonucleotides (1×10^6 dpm/mL) as described above or with cDNA probes (5×10^5 dpm/mL) as specified by Wahl et al. (1979). Dextran sulfate (5%) was included in the latter hybridization. Probes were radiolabeled by nick translation in the presence of [α-^{32}P]NTPs (ATP, GTP, CTP, and TTP, ~3000 Ci/mmol; Amersham Corp.), with DNA polymerase I (Rigby et al., 1977). Nonspecific binding was eliminated by washing with 2× SSC–0.1% SDS and 0.1× SSC–0.1% SDS at the hybridization temperatures. Specific binding was detected by radioautography.

Isolation and Sequencing of cDNA Fragments. A human liver λgt11 cDNA library (Kwok et al., 1985) was kindly provided by Dr. Vincent Kidd (University of Alabama, Birmingham, AL). The library (7×10^5 plaque forming units, PFU) was screened independently with the apoJα and apoJβ oligonucleotide probes. Recombinant phage were transferred to nitrocellulose and fixed by baking at 80 °C under vacuum (Ausubel et al., 1989). Hybridization conditions were identical with those described for hybridization of oligonucleotides to RNA. Positive phage, confirmed in four consecutive screenings, were plaque purified, and the DNA was isolated (Maniatis et al., 1982a). The λgt11 clone 1-3 selected for sequence analysis was digested with *Eco*RI, and the cDNA insert purified by low-melt agarose gel electrophoresis (Parker & Seed, 1980) and subcloned into M13mp18 or mp19 (Messing, 1983; Norrander et al., 1983) for sequence analysis. When necessary, the inserts were digested with a restriction enzyme, and the resulting fragments were isolated from low-melt agarose gels (Parker & Seed, 1980). Fragments not having cohesive ends were made blunt with the large fragment of *Escherichia coli* polymerase I (Wartell & Reznikoff, 1980) and cloned into appropriate sites. M13 single-stranded DNA was purified (Amersham Corp., 1983) and sequenced by the dideoxy methodology (Sanger et al., 1977), using both Klenow and T7 polymerase (Sequenase Version 2.0, U.S. Biochemical Corp.) in the presence of [α-^{32}P]ATP (~800 Ci/mmol; Amersham Corp.).

Genomic Southern Hybridization. Genomic DNA was isolated from human spleen (Maniatis et al., 1982b). Genomic DNA from human peripheral blood mononuclear cells was provided by Dr. Marcia Shull (Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati, Cincinnati, OH). Mouse genomic DNA was provided by Dr. Steven Potter (Childrens Hospital Research Foundation, Cincinnati, OH). Genomic DNA was digested to completion with *Eco*RI or *Pvu*II, and 20 μg of the DNA was electrophoresed through 0.8% agarose and transferred to a Hybond nylon membrane (Amersham Corp.) (Southern, 1975) DNA was cross-linked to the membrane by ultraviolet irradiation. Unoccupied sites were blocked at 42 °C for 1 h with 6× SSC, 0.1% SDS, 0.2% PVP, BSA, and Ficoll, 5% dextran sulfate, and 100 μg/mL denatured salmon sperm DNA. The blot was hybridized at 42 °C with 1×10^6 dpm/mL probe derived from 733 bp encoding the 5′ end of the apoJ cDNA in 6× SSC, 50% formamide, 5% dextran sulfate, 1% SDS, and 100 μg/mL salmon sperm DNA. Nonspecific binding was eliminated by washing at 42 °C with 2× SSC–0.1% SDS.

Results

ApoJα and ApoJβ Oligonucleotides Hybridize with a 1.9-kb Message. HepG2 cells synthesize and secrete apoJ (de Silva, 1989), and apoJ is produced from HepG2 mRNA translated in vitro (de Silva et al., 1990b). To establish the size of the mRNA species encoding the α and β subunits of apoJ, mRNA was isolated from confluent HepG2 cells, electrophoresed,

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          1                                   10                                  20
APOJα     ASP GLN THR VAL SER ASP ASN GLU LEU GLN GLU MET SER ASN GLN GLY SER LYS TYR VAL ASN LYS GLU ILE HIS ASN-
OLIGO 3'  CTI GTC TGI CAC AGI CTI TTI CTC GAC GTC CTC TAC AGI TTI GTC CCI AGI TTC ATI CAC TTI TTC CTC TA 5'

          1                                   10                                  20
APOJβ     SER LEU MET PRO PHE SER PRO TYR GLU PRO LEU ASN PHE HIS ALA MET PHE GLN PRO PHE LEU GLU MET ILE HIS GLU-
OLIGO 3'          TAC GGI AAI AGI GGI ATI CTC GGI GAC TTI AAI GTI CGI TAC AAI GTC GGI AAI GAC CTC TAC TAI GTI CT 5'
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FIGURE 1: ApoJα and apoJβ oligonucleotide probes. Oligonucleotides, 71 bases in length, were synthesized according to the apoJα and apoJβ amino-terminal sequences as described under Materials and Methods.

transferred to nitrocellulose, and incubated with oligonucleotide probes (Figure 1) specific for apoJα and apoJβ. These probes were synthesized to correspond to the amino-terminal sequences of apoJα and apoJβ (de Silva et al., 1990b). The apoJα and apoJβ oligonucleotides each hybridized to a single mRNA of 1.9 kb. With comparable end labeling, a weak hybridization signal was obtained with the apoJα oligonucleotide compared to that with the apoJβ oligonucleotide. The fact that both oligonucleotides hybridized with HepG2 mRNA indicated that the probes could be used to screen a cDNA library.

Isolation and Analysis of ApoJ cDNA. A human liver λgt11 cDNA library containing 28 million independent recombinants at a density of 5×10^4 bacteriophages/plate was screened independently with the apoJα and apoJβ oligonucleotide probes. In the initial screening, five recombinants hybridized with the apoJα oligonucleotide, and seven recombinants hybridized with the apoJβ oligonucleotide. In three subsequent screenings, four of the apoJα clones and all of the apoJβ clones were confirmed as positives. Southern blot analysis of all 11 clones with the apoJα and apoJβ oligonucleotides revealed that the four identified with the apoJα oligonucleotide also hybridized with the apoJβ oligonucleotide. The longest of these four clones, λ1-3, was sequenced. λ1-3 was digested with *Eco*RI, and the insert was recovered in two fragments of 733 and 827 bp, indicating the presence of an internal *Eco*RI site. These fragments were subcloned into M13mp19; fragments inserted in both orientations were isolated and sequenced. The sequence strategy is illustrated in Figure 2.

Although subclones of the 3′ fragment were obtained in both orientations, difficulties in sequencing through the poly(A) tail were encountered. Consequently, a 20-base oligonucleotide corresponding to residues 1170–1190 was synthesized and used as a sequencing primer. To sequence through the internal *Eco*RI site of the apoJ clone, λ1-3 was double digested with *Ava*I (at base 506) and *Bam*HI (at base 1417). The resulting 911-bp fragment was blunt ended with Klenow and subcloned into *Sma*I-digested M13mp18. This fragment was sequenced, using both the universal sequencing primer at base −40 and a sequence-specific primer corresponding to bases 639–659 (Figure 2).

The locations of the regions in the cDNA which hybridized with the apoJα and apoJβ oligonucleotides are designated by solid boxes in Figure 2. A region homologous to the apoJα oligonucleotide (>90%, excluding inosine substitutions) was identified at the 5′ end (16–66 bp). A region of homology to the apoJβ oligonucleotide (>90%) was identified in the middle of the clone (604–675 bp).

ApoJα and ApoJβ Are Synthesized in Tandem as a Single Translation Product. The sequence of the cDNA and the deduced amino acid sequence of apoJ are presented in Figure 3. The cDNA is incomplete at the 5′ end. On the basis of a comparison with the deduced protein sequence, the 5′ end of apoJ cDNA begins with methionine-12 of apoJα. The first 15 bases at the 5′ end are artifacts of library construction. A

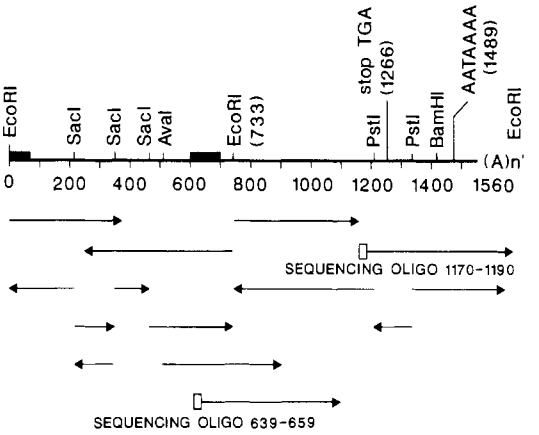

FIGURE 2: Sequence strategy. A human fetal liver λgt11 cDNA library was screened with end-labeled apoJα and apoJβ oligonucleotides as described under Materials and Methods. Clone λ1-3 was digested with restriction endonucleases, and the fragments were subcloned and sequenced as illustrated. Sequencing details are specified under Materials and Methods and under Results.

TGA in-frame stop codon is present at base 1266, delineating the 3′ end of the open reading frame; a 244-bp untranslated 3′ region follows. A canonical polyadenylation signal, AATAAAA, is present at base 1489. The clone contains 50 adenosines of the poly(A) tail. The cDNA was confirmed to encode apoJ by comparison of the deduced protein sequence with actual protein sequence obtained for the amino termini (de Silva et al., 1990b) of apoJα and apoJβ (bold underlines in Figure 3) and for proteolytic fragments of these subunits (thin underlines) determined in this investigation. To date, 32% of the protein sequence has been determined experimentally, all of which is in agreement with the predicted sequence except amino acid 30 of apoJα, which was determined to be glutamine by amino acid sequencing and glycine by cDNA analysis.

The sequences encoding apoJα and apoJβ are present linearly on a single cDNA clone, indicating that the proteins are synthesized in tandem as a single primary translation product. The cDNA, together with amino-terminal residues 1–11 of apoJα, predicts a mature protein (minus any signal peptide) of 427 amino acids with a calculated molecular mass of 50088 daltons. The amino-terminal sequences of apoJα and apoJβ correspond to amino acids 1–30 and 206–236, respectively, of the primary translation product. These sequences are highlighted by bold lines in Figure 3. Serine-206 corresponds to amino acid 1 of mature apoJβ, indicating that apoJα and apoJβ are produced by a proteolytic cleavage at the carboxyl end of arginine-205. ApoJα is 205 amino acids in length with a calculated molecular mass of 24 201 daltons; apoJβ is 222 amino acids in length with a calculated molecular mass of 25 887 daltons. The calculated molecular masses are in good agreement with the values determined by electrophoretic analysis of chemically deglycosylated apoJα and apoJβ of 24 and 25 kDa, respectively (de Silva et al., 1990b). Moreover, the amino acid compositions of the subunits calculated from

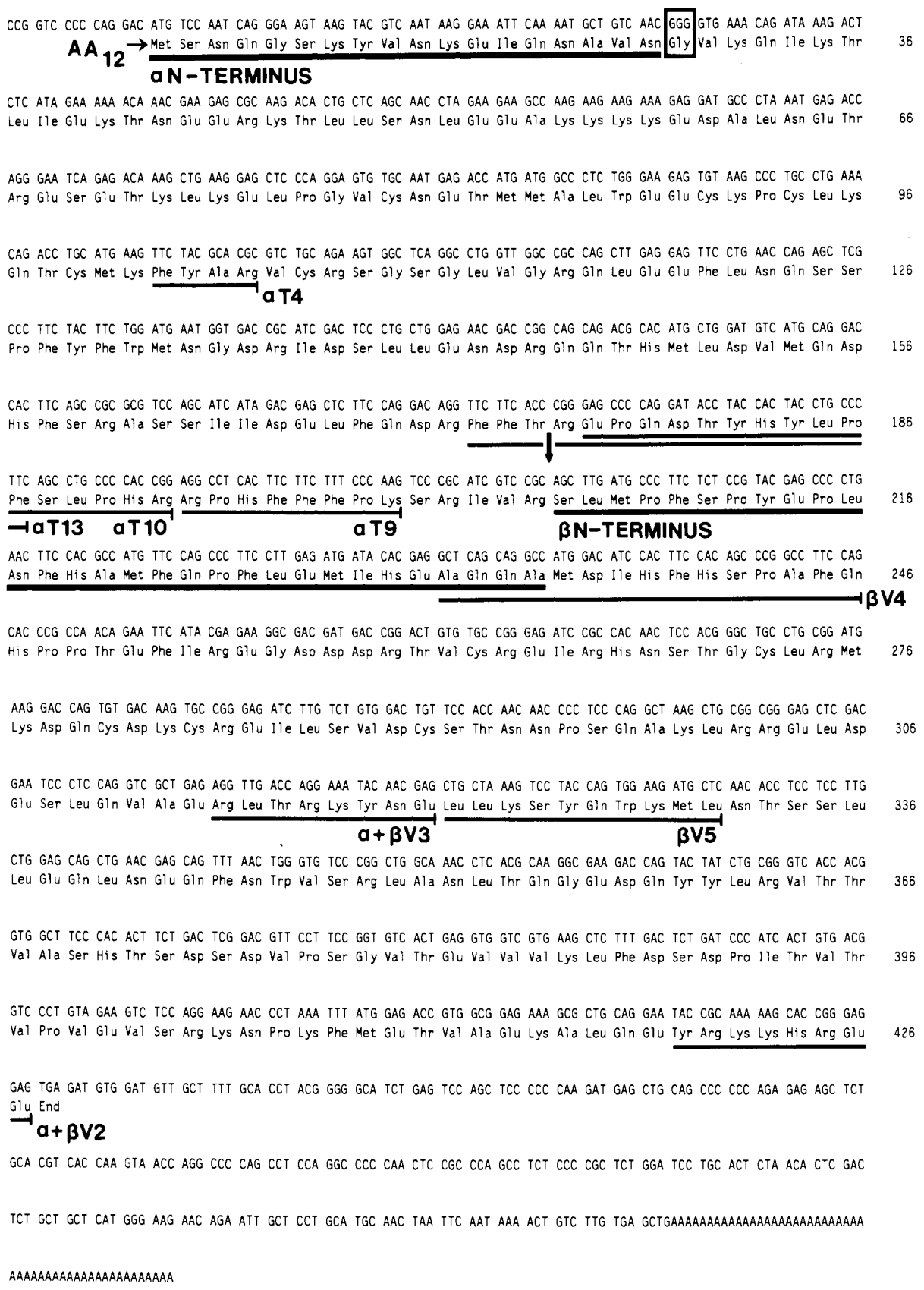

FIGURE 3: cDNA and deduced amino acid sequences of apoJ. The cDNA sequence (top line), numbered on the left margin, and the amino acid sequence (bottom line) deduced from the cDNA, numbered on the right margin, are illustrated. Bold lines indicate the match of the amino-terminal protein sequence of apoJα and apoJβ with the deduced sequence. The thin lines indicate matches between the deduced sequence and peptide sequences of apoJα (α) and apoJβ (β) obtained by amino acid sequencing of peptides produced by trypsin (T) and *S. aureus* V8 protease (V) hydrolysis of the apoJ subunits. Specific peptide numbers are identified. The arrow preceding the first amino acid (serine-206) of apoJβ indicates the probable cleavage site that generates mature apoJα and apoJβ. The glycine-30 indicated by the box was determined as glutamine by protein sequencing.

Table I: Interesting ApoJ Sequences

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Cysteine-Rich Domains:

            80                  90                 100                 110
apoJα     - V C N E T M M A L W E E C K P C L K Q T C M K F Y A R V C R S -

apoJβ     - V C R E I R H N S T G C L R M K D Q C D K C R E I L S V D C S -
                        270                 280                 290

Potential Heparin-Binding Domains:

                    50                  60
apoJα     - R K T L L S N L E E A K K K K E D -
          190       196
apoJα     - P H R R P H F

          402       408
apoJβ     - S R K N P K F -
          420         427
apoJβ     - Y R K K H R E E (COOH)
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the sequence agree with the values determined experimentally.

The ApoJ Gene Is Present in the Human and Mouse Genome. Southern analysis was used to assess whether, in the human and mouse genomes, the apoJ gene is a part of a large gene family. Genomic DNA was digested with *Eco*RI or *Pvu*II, electrophoresed, transferred to a nylon membrane, and hybridized with a 733-bp fragment derived from the 5′ end of the apoJ cDNA (Figure 4). The apoJ cDNA contained one *Eco*RI site at position 733 and one *Pvu*II site at position 997. Hybridization of human spleen *Eco*RI-digested DNA (human 1) with the 733-bp probe revealed four fragments of 3.47, 2.95, 3.34, and 1.07 kb. Four fragments of 1.69, 1.41, 1.14, and 0.6 kb were identified in *Pvu*II-digested DNA; the 0.6-kb fragment appeared to be a doublet, and the 1.69-kb signal was weak. DNA was isolated from a different individual (human 2), digested with *Pvu*II, and analyzed. In this case, three fragments of 1.7, 1.47, and 1.14 kb were identified by hybridization. The differences between the two human subjects in hybridization patterns following *Pvu*II digestion suggest that there may be a polymorphism at the *Pvu*II site.

In contrast to the results with human DNA, single bands of 8.7 and 6.9 kb, respectively, were detected in mouse DNA digested with *Eco*RI or *Pvu*II. Comparison of the relative intensities of hybridization of plasmid DNA and genomic DNA, taking into consideration the number of genomes represented, suggests that in both human and mouse genomes apoJ is present in either one or only a few copies.

ApoJ mRNA Has a Wide Tissue Distribution. To determine the tissue distribution of apoJ mRNA, RNA was isolated from a variety of human tissues, and the presence of the apoJ message was assessed by Northern blot analysis. Total RNA was hybridized with the 5′ 733-bp cDNA fragment. A 1.9-kb message that was detected in the HepG2 mRNA was evident in a number of tissues (Figure 5). High levels of the message were detected in the steroidogenic tissues, testis and ovary, and also in brain. In addition, apoJ mRNA was detected in heart, liver, lung, and spleen. A weak hybridization signal was detected in breast. The synthesis and translation of apoJ mRNA in breast tissue were confirmed by analysis of human milk. ApoJ was identified in milk by electroimmunoblotting with the apoJ mAb11 (Stuart, unpublished data). Peripheral blood T-lymphocytes had no detectable apoJ mRNA.

Secondary Structure Analysis of ApoJ Predicts Lipid- and Heparin-Binding Domains. The secondary structure of apoJ was predicted (Figure 6) in order to identify regions of apoJ that may constitute functional domains. Chou–Fasman (1974)

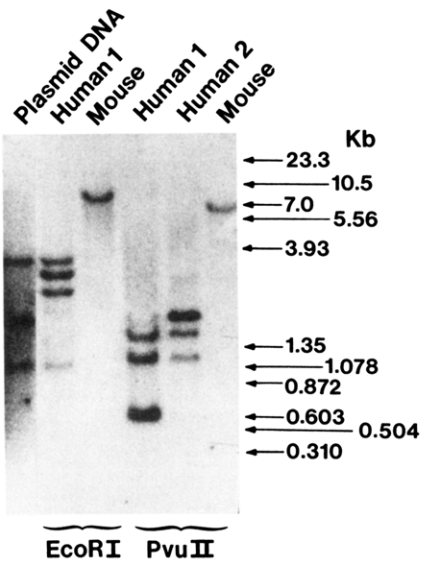

FIGURE 4: Analysis of human and mouse genomic DNA, using apoJ cDNA. Human DNA (20 μg), isolated from spleen (human 1) and from human peripheral blood mononuclear cells (human 2), and mouse DNA (20 μg) were digested with *Eco*RI or *Pvu*II. Plasmid DNA (0.0001 μg), containing the 5′-end *Eco*RI fragment of apoJ, was included as a control. DNA samples were electrophoresed through a 0.8% agarose gel, transferred to a nylon membrane, and hybridized with the nick-translated 5′ 733-bp *Eco*RI fragment of the apoJ cDNA as described under Materials and Methods. Reference molecular weight standards were *Bam*HI-digested Charon 4A and *Hae*III-digested φX174 phage; the DNA was visualized with ethidium bromide.

analysis of apoJ predicted 35% α-helix, 20% β-sheet, and 44% random structure. Analysis by the method of Garnier et al. (1978) predicted that apoJ consists of 42% α-helix, 13% β-sheet, 22% β-turn and 21% random structure. Closer examination of the predicted α-helical regions indicated that three of these could generate amphiphilic helices, and they are displayed as helical wheel diagrams (Schiffer & Edmundson, 1967) in Figure 7. The amphiphilic helix is thought to play an important role in the interaction of apolipoproteins with lipid (Segrest et al., 1974; Kaiser & Kézdy, 1983). One potential lipid-binding domain was present in apoJα (residues 150–167), and two were present in apoJβ (residues 221–237 and 401–418).

Other interesting structural motifs are present in the apoJ sequence. Both apoJα and apoJβ contain five cysteine residues, and these are clustered in a 30-residue domain near the middle of each sequence (Table I). Since the pattern of disulfide

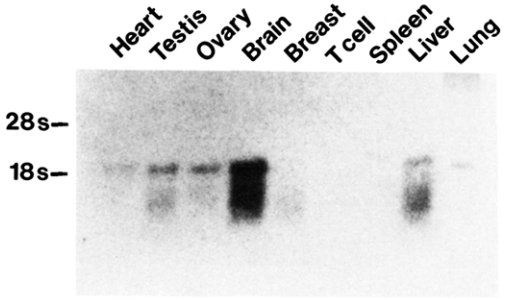

FIGURE 5: Analysis of the distribution of apoJ mRNA in human tissues. RNA (5 μg) from human tissues, isolated as described under Materials and Methods, was electrophoresed through a 0.7% agarose gel containing 5 mM methylmercury, transferred to DBM paper, and hybridized with the nick-translated 5′ 733-bp *Eco*RI fragment of apoJ cDNA. The 18S and 28S ribosomal RNA, detected by ethidium bromide staining, are indicated.

bonding has an important influence on protein folding, it will be important to determine which cysteines are involved in disulfide bonds and whether the bonds are interchain, intrachain, or both. Additionally, apoJ contains short stretches of the basic amino acids lysine and arginine. In four of these regions (Table I), the organization of the basic residues closely approximates the consensus sequence that is important in protein–heparin interactions (Cardin & Weintraub, 1989). In addition, five very short segments in apoJα are repeated in apoJβ (Table II). These regions of identity may account for the fact that two mAb, mAb11 and mAb5, recognize protein epitopes in both apoJα and apoJβ (de Silva et al., 1990b).

Table II: Sequence Repeats in ApoJα and ApoJβ

sequence	apoJ location	sequence	apoJ location
NEL	apoJα 7–9	SLLE	apoJα 139–142
	apoJβ 320–322		apoJβ 335–338
LNE	apoJα 63–65	PFS	apoJα 186–188
	apoJβ 340–342		apoJβ 209–211
VCR	apoJα 106–108		
	apoJβ 262–264		

Human ApoJ Is Homologous to Rat SPG2. A search of the NIH Genbank revealed that apoJ has a high degree of homology to the major rat Sertoli cell protein, sulfated gly-

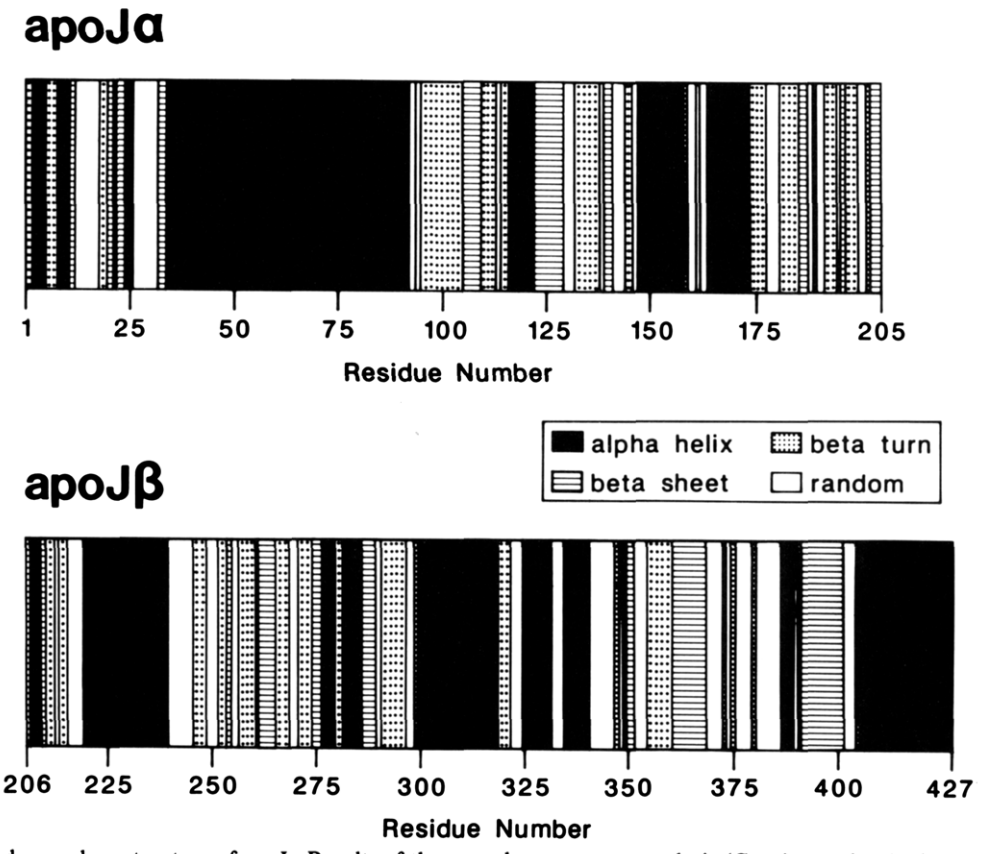

FIGURE 6: Predicted secondary structure of apoJ. Results of the secondary structure analysis (Garnier et al., 1978), using DNANALYZE, are illustrated.

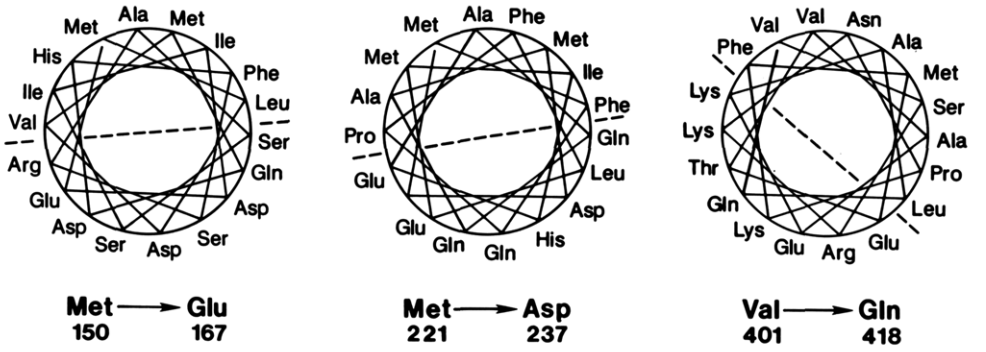

FIGURE 7: Predicted amphiphilic helices of apoJ. Helical wheel diagrams (Schiffer & Edmondson, 1967) of apoJ sequences are illustrated: Met-150 to Glu-167 in apoJα; Met-221 to Asp-237 and Val-401 to Gln-418 in apoJβ.

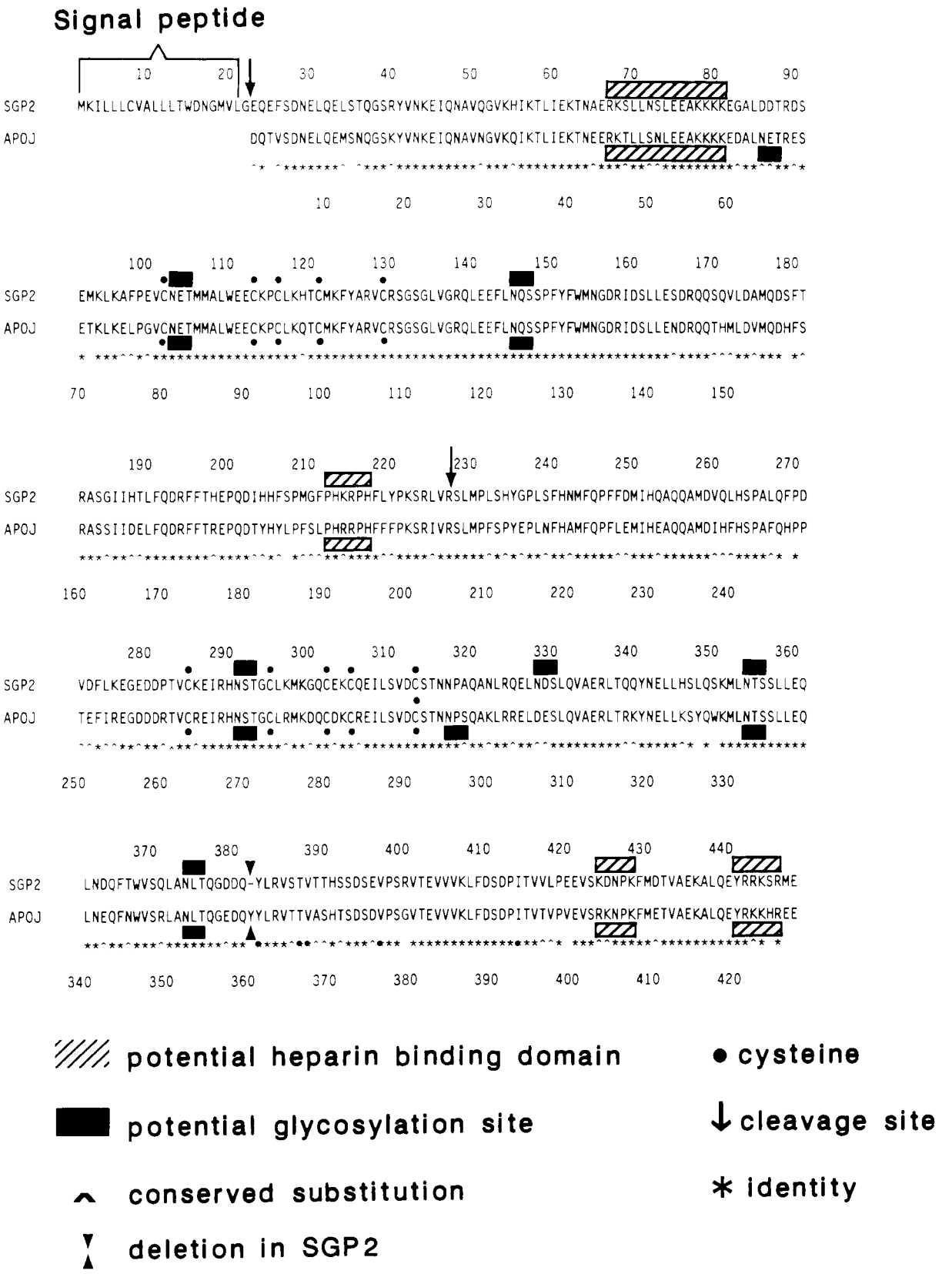

FIGURE 8: Comparison of the amino acid sequences of human apoJ and rat SGP2. The sequence of SGP2 (Collard & Griswold, 1987), deduced from its cDNA, is shown on the top line. The sequence of apoJ, deduced from the cDNA sequence and including amino-terminal amino acids 1–11 determined by amino acid sequencing of apoJα, is shown on the bottom line.

coprotein 2 (SGP2) (Collard & Griswold, 1987). SPG2, like apoJ, is a disulfide-linked heterodimer, and the subunits are produced by cleavage of a single primary translation product. The overall identity between apoJ and SPG2 is 77.2%, suggesting that apoJ is the human analogue of SGP2. The complete SGP2 sequence, including its signal peptide of 22 amino acids, is compared with that of apoJ in Figure 8. Mature SGP2 consists of 426 amino acids; apoJ has 427 amino acids with an additional tyrosine at position 360. The proposed cleavage site between arginine-205 and serine-206 of apoJ is present in SGP2 and has also been suggested (Collard & Griswold, 1987) to be the site of SGP2 cleavage to produce

the mature SGP2 subunits. Both SGP2 and apoJ are extensively glycosylated (de Silva et al., 1990b; Sylvester et al., 1984; Griswold et al., 1986). Seven potential N-linked glycosylation sites exist in the apoJ sequence, on the basis of the proposed consensus Asn-X-Ser/Thr (Marshall, 1974). Six of these potential glycosylation sites are also present in SGP2. Both apoJα and apoJβ have 5 cysteines that are clustered within a region of 30 amino acids (Table I), and these cysteine domains are present in SGP2, as are the potential heparin-binding sites (Table I).

Discussion

The primary structure of apoJ was determined by the combined approaches of protein chemistry and molecular biology. The structure of apoJ, a disulfide-linked heterodimer, is clearly distinct from that of other human plasma apolipoproteins which have been described to date. The primary structure provides an unequivocal answer to the question of the relationship between the apoJ subunits. As suggested previously (de Silva et al., 1990b), apoJα and apoJβ are produced from a common apoJ precursor protein. The cDNA sequence reveals that they are synthesized in tandem as a single protein of 50 kDa. Mature apoJα and apoJβ are produced by a posttranslational cleavage at the carboxyl end of Arg-205 of apoJ. The amino-terminal 205 amino acids of apoJ become apoJα; the carboxy-terminal 222 amino acids, apoJβ. The molecular masses of deglycosylated apoJα and apoJβ, 24 and 28 kDa, respectively, agree well with their calculated molecular masses of 24 201 and 25 877 daltons. The relationship between apoJα and apoJβ explains other structural data. When isolated from human plasma, more than 95% of the apoJ has been cleaved, and apoJ consists of approximately equal amounts of each subunit (de Silva et al., 1990b). Once synthesized and cleaved, the resulting subunits are maintained as a single unit, suggesting that the disulfide bonds are formed prior to proteolysis. Although their amino acid compositions suggest extensive similarities between apoJα and apoJβ, they are distinct. The existence of five sequence repeats of three or four amino acids each in apoJα and apoJβ may account for the fact that two apoJ mAb recognize both apoJα and apoJβ.

ApoJ shows striking homology, 78.9% at the nucleic acid level and 77.2% at the amino acid level, to the major rat Sertoli cell product SGP2 (Collard & Griswold, 1987), also called clusterin in the ram (Cheng et al., 1988). SGP2 is a dimeric acidic glycoprotein that accounts for as much as 50% of the Sertoli cell secreted protein. The molecular mass of the SGP2 heterodimer is 70 kDa, and its p*I* is 4.6. One subunit has a molecular mass of 47 kDa and a p*I* of 3.7–4.5; the other, a molecular mass of 33 kDa and a p*I* of 4.8–5.4. Like apoJα and apoJβ, the SGP2 subunits are produced by hydrolysis of an internal Arg–Ser bond. The extensive homology between the two proteins suggests that apoJ is the human counterpart of rat SGP2. This supposition is supported not only by the sequence homology but also by the conservation of both real and predicted functional domains, e.g., the Arg–Ser cleavage site, the cysteine-rich domains, and the putative lipid-binding, glycosylation, and heparin-binding domains. Moreover, apoJ and SGP2 have a broad tissue distribution in human and rat, respectively. SGP2 mRNA is present in all rat tissues studied. The message is most abundant in testis, epididymis, liver, and brain with lower mRNA levels in kidney, spleen, and mammary gland (Collard & Griswold, 1987). The level of human apoJ mRNA is highest in brain followed by testis, ovary, liver, heart, and lung; low levels of apoJ mRNA are present in spleen and mammary gland. The argument that apoJ is the human analogue of rat SGP2 is strengthened by the finding that apoJ is encoded by a single-copy gene in mouse and perhaps also in human. Although three to four small genomic fragments that hybridized with the apoJ cDNA were present in the human genome by Southern analysis, the sum of the molecular weights of these fragments is roughly the same as the size of the single band in the mouse genome that hybridizes with the apoJ cDNA. We therefore propose that there is a single apoJ gene in the human genome and that this gene has internal restriction sites for *Eco*RI and *Pvu*II. We know from this work that the cDNA has an internal *Eco*RI site.

As an apolipoprotein in a human plasma HDL, apoJ presumably functions to solubilize and transport lipid in the plasma compartment. It may have other functions as well. The physiological role of SGP2 has not yet been delineated. It has been suggested that SGP2 functions as an immunosuppressive agent and/or a transporter of hydrophobic compounds such as lipids and fatty acids (Griswold, 1988). A lipid transport function is consistent with the abundance of each protein; the amount of apoJ in human plasma is estimated to be 9 mg/dL (de Silva et al., 1990a); that of SGP2, as much as 50% of the product secreted by rat Sertoli cells. The role of apoJ, and by inference SGP2, in lipid transport is supported by the structural analysis. ApoJ is predicted to consist of 42% α-helix and 13% β-sheet, important motifs in the interaction of proteins at the lipid–water interface (Kaiser & Kézdy, 1983). Three of the putative α-helical regions of apoJ can form amphiphilic helices, one large helical domain in apoJ and two smaller domains in apoJβ. Amphiphilic helices are responsible for the interaction of apolipoproteins with phospholipid molecules to stabilize phospholipid–neutral lipid emulsions (Segrest et al., 1974).

Accepting a role for apoJ in lipid transport, apoJ has intriguing similarities with another apolipoprotein involved in lipid transport, apoE. While the similarities are not reflected at the level of the primary sequence, apoE and the apoJ subunits share lipid-binding and heparin-binding motifs. ApoE and the apoJ subunits are intermediate in the size range of apolipoproteins, between the apoC's and apoB, and they have similar isoelectric points. ApoE has 299 amino acids, a molecular mass of 34 kDa, and a p*I* of 5.7–6.2 (Rall et al., 1986). ApoJα has 205 amino acids, a nonglycosylated molecular mass of 24 kDa, and a p*I* of 5.9; apoJβ has 222 amino acids, a nonglycosylated molecular mass of 28 kDa, and a p*I* of 6.1 (de Silva et al., 1990b). The amounts of both proteins in the plasma of fasted subjects are comparable. More interesting are the similar patterns of tissue distribution of the message. ApoE mRNA or protein has been detected in testis, ovary, adrenals, brain, intestine, pancreas, heart, stomach, lung, liver, and kidney and in macrophages (Driscoll & Getz, 1984; Elshourbagy et al., 1985; Lin-Lee et al., 1985; Williams et al., 1985; Zannis et al., 1985). Although some of these tissues have not been examined for apoJ, its mRNA exists in every tissue that contains the apoE mRNA. Thus both apoE and apoJ can exist in the plasma compartment and in compartments that are separated from the plasma compartment by a physiological barrier, e.g., the blood–brain and blood–testis barriers. These apolipoproteins therefore not only can serve to transport lipids between organs but can facilitate lipid transport among cells within an organ or tissue (Mahley, 1988). Localized redistribution of lipids by apoE, for example, has been documented (Boyles et al., 1989) to occur in the nervous system following injury and during regeneration of peripheral neurons.

We propose that apoE and apoJ solubilize and transport lipids within tissues, with the target cell specificity determined

at the receptor level. ApoE delivers lipids to cells by first binding to receptors on the cell membrane. ApoE, like apoB100, can target lipoproteins to the LDL receptor (Mahley, 1988), to the chylomicron receptor (Hui et al., 1984), and perhaps to other apoE receptors (Herz et al., 1988). If apoJ recognizes specific cell membrane receptors, they must be distinct from those for apoE since there is little similarity between the primary structures of the two apolipoproteins. Receptor recognition of apoJ is indicated by the strong interaction between the rat homologue of apoJ, SGP2, and sperm membranes, an interaction that requires detergent disruption. SGP2 is concentrated on the acrosome and tail regions of mature rat sperm, and Griswold (1988) has suggested that the protein facilitates sperm maturation. The interaction between apoE and the LDL receptor is mediated by sequences in apoE that are part of or proximal to a heparin-binding domain (Mahley, 1988; Cardin & Weintraub, 1989). By analogy, apoJ may interact with cell surfaces via its heparin-binding sequences. Three of these domains, residues 190–196, 402–408, and 420–427, are close to or overlap the predicted amphiphilic helices at residues 221–237 and 401–427. The proximity of these putative functional domains predicts that apoJ may not simultaneously bind lipid and glycosaminoglycans/receptors, suggesting a lipid "dumping" mechanism.

The delineation of the primary structure and the tissue distribution of apoJ has provided insight about the possible functions of this apolipoprotein. Among apolipoproteins, apoJ is unique in structure. ApoJ is comprised of two subunits, apoJα and apoJβ, which are synthesized as a 427 amino acid precursor and then posttranslationally cleaved at an internal Arg–Ser bond. The two subunits remain associated by disulfide bonds. The high degree of similarity of human apoJ to rat SGP2 indicates that the two proteins are probably the same, with the small differences due to differences between species. On the basis of similar tissue distributions and similar overall structure motifs, apoJ and apoE are postulated to transport lipids within the same tissues but to different cells and/or in response to different stimuli.

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REFERENCES

Alwine, J. C., Kemp, D. J., & Stark, G. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5350–5354.

Amersham Corp. (1983) in *M13 Cloning and Sequencing Handbook*, Amersham Corp., Arlington Heights, IL.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., & Struhl, K. (1989) in *Current Protocols in Molecular Biology*, Vol. 1, Section 6, Wiley, New York.

Aviv, H., & Leder, P. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1408–1412.

Bailey, J. M., & Davidson, N. (1976) *Anal. Biochem. 70*, 75–85.

Boyles, J. K., Zoellner, C. D., Anderson, L. J., Kosik, L. M., Pitas, R. E., Weisgraber, K. H., Hui, D. Y., Mahley, R. W., Gebicke-Haerter, P. J., Ignatius, M. J., & Shooter, E. M. (1989) *J. Clin. Invest. 83*, 1015–1031.

Cardin, A. D., & Weintraub, H. J. R. (1989) *Arteriosclerosis 9*, 21–32.

Castelli, W. P., Doyle, J. T., Gordon, T., Hames, C. G., Hjortland, M. C., Hulley, S. B., Kagan, A., & Zukel, W. J. (1977) *Circulation 55*, 767–772.

Cheng, C. Y., Chen, C.-L., Feng, Z. M., Marshall, A., & Bardin, C. W. (1988) *Biochem. Biophys. Res. Commun. 155*, 398–404.

Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., & Rutter, W. J. (1979) *Biochemistry 18*, 5294–5299.

Chomczynski, P., & Sacchi, N. (1987) *Anal. Biochem. 162*, 156–159.

Chou, P. Y., & Fasman, G. D. (1974) *Biochemistry 13*, 222–245.

Collard, M. W., & Griswold, M. D. (1987) *Biochemistry 26*, 3297–3303.

de Silva, H. V. (1989) Ph.D. Thesis, University of Cincinnati.

de Silva, H. V., Stuart, W. D., Duvic, C. R., Wetterau, J. R., Ray, M. J., Ferguson, D. G., Albers, W. H., Smith, W. R., & Harmony, J. A. K. (1990a) *J. Biol. Chem.* (in press).

de Silva, H. V., Stuart, W. D., Park, Y. B., Mao, S. J. T., Busch, S. J., Gil, C. M., & Harmony, J. A. K. (1990b) *J. Biol. Chem.* (in press).

Driscoll, D. M., & Getz, G. S. (1984) *J. Lipid Res. 25*, 1368–1379.

Elshourbagy, N. A., Liao, W. S., Mahley, R. W., & Taylor, J. M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 203–207.

Garnier, J., Osguthorpe, D. J., & Robson, B. (1978) *J. Mol. Biol. 120*, 97–120.

Glomset, J. A. (1968) *J. Lipid Res. 9*, 155–167.

Griswold, M. D. (1988) *Int. Rev. Cytol. 110*, 133–156.

Griswold, M. D., Roberts, K., & Bishop, P. (1986) *Biochemistry 25*, 7265–7270.

Herz, J., Hamann, U., Rogne, S., Myklebost, O., Gauespohl, H., & Stanley, K. K. (1988) *EMBO J. 7*, 4119–4127.

Hui, D. Y., Innerarity, T. L., Milne, R. W., Marcel, Y. L., & Mahley, R. W. (1984) *J. Biol. Chem. 259*, 15060–15068.

Kaiser, E. T., & Kézdy, F. J. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1137–1143.

Kane, J. P. (1986) *Adv. Exp. Med. Biol. 201*, 29–35.

Knowles, B. B., Howe, C., & Aden, D. P. (1980) *Science 209*, 497–499.

Krauss, R. M., & Nichols, A. V. (1986) *Adv. Exp. Med. Biol. 201*, 17–27.

Kwok, S. C. M., Ledley, F. D., DiLella, A. G., Robson, K. J. H., & Woo, S. L. C. (1985) *Biochemistry 24*, 556–561.

Lathe, R. (1985) *J. Mol. Biol. 183*, 1–12.

Lin-Lee, Y.-C., Kao, F.-T., Cheung, P., & Chan, L. (1985) *Biochemistry 24*, 3751–3756.

Mahley, R. W. (1988) *Science 240*, 622–630.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982a) in *Molecular Cloning, a Laboratory Manual*, pp 63–85, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982b) in *Molecular Cloning, a Laboratory Manual*, pp 280–281, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Maniatis, T., Hardison, C. R., Lacy, E., Lauer, J., O'Connell, C., & Quon, D. (1978) *Cell 15*, 687–701.

Marshall, R. D. (1974) *Biochem. Soc. Symp. 40*, 17–26.

Maxam, A. M., & Gilbert, W. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 560–564.

Messing, J. (1983) *Methods Enzymol. 101*, 20–79.

Norrander, J., Kempe, T., & Messing, J. (1983) *Gene 26*, 101–106.

Ohtsuka, E., Matsuki, S., Ikehara, M., Takahashi, Y., & Matsubara, K. (1985) *J. Biol. Chem. 260*, 2605–2608.

Parker, R. C., & Seed, B. (1980) *Methods Enzymol. 65*, 358–371.

Rall, S. C., Jr., Weisgraber, K. H., & Mahley, R. W. (1986) *Methods Enzymol. 128*, 237–287.
Rigby, P. W. J., Dieckmann, M., Rhodes, C., & Berg, P. (1977) *J. Mol. Biol. 113*, 237–251.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.
Schiffer, M., & Edmundson, A. B. (1967) *Biophys. J. 7*, 121–135.
Segrest, J. P., Jackson, R. L., Morrisett, J. D., & Gotto, A. M., Jr. (1974) *FEBS Lett. 38*, 247–253.
Southern, E. M. (1975) *J. Mol. Biol. 98*, 503–517.
Sylvester, S. R., Skinner, M. K., & Griswold, M. D. (1984) *Biol. Reprod. 31*, 1087–1101.
Wahl, G. M., Stern, M., & Stark, G. R. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3683–3687.
Wartell, R. M., & Reznikoff, W. S. (1980) *Gene 9*, 307–319.
Wernke, G. R., & Thompson, R. L. (1989) *Biophys. J. 55*, 371.
Williams, D. L., Dawson, P. A., Newman, R. C., & Rudel, L. L. (1985) *J. Biol. Chem. 260*, 2444–2451.
Zannis, V. I., Cole, F. S., Jackson, C. L., Kurnit, D. M., & Karathanasis, S. K. (1985) *Biochemistry 24*, 4450–4455.

Structural and Functional Changes Associated with Modification of the Ubiquitin Methionine

Shakti Bamezai, Mary Ann T. Banez, and Esther Breslow*

Department of Biochemistry, Cornell University Medical College, New York, New York 10021
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ABSTRACT: The effects of oxidation and cleavage of Met-1 of ubiquitin on conformation and biological activity were individually investigated. Proton NMR studies demonstrated that oxidation to the sulfone led to restricted structural perturbations at neutral pH, particularly in the vicinity of Ile-61. Below pH 3, in the presence of acetic acid, oxidation to the sulfone facilitated a conformational expansion demonstrable by retardation on gel electrophoresis and CD changes below 210 nm. The predominant phase of the low-pH transition did not involve significant changes in α-helix content, indicating the capacity of ubiquitin for limited structural transitions. Cleavage of Met-1 by CNBr, on the other hand, was associated with a global unfolding transition below pH 4 that involved a major loss of α-helix. Differences in the behavior of the native and des-Met proteins at low pH indicate that Met-1 contributes a minimum of 3.4 kcal/mol to the stability of the native conformation. Two Met-1 sulfoxide isomers, of markedly different conformational stability, were formed by treatment of ubiquitin with H_2O_2. One isomer was similar in stability to the sulfone, while the other was intermediate in stability between the sulfone and des-Met proteins, the differences potentially interpretable in terms of the geometry of the Met-1–Lys-63 hydrogen bond. The overall activities of the oxidized and des-Met derivatives in ATP-dependent proteolysis differed subtly from that of native ubiquitin. The unresolved sulfoxides exhibited an approximately 50% increase in activity, while the sulfone and des-Met proteins exhibited a 50% decrease in activity at low concentrations and normal activity at higher concentration. The results demonstrate that Met-1 is not essential to ubiquitin activity, but allow the possibility that it influences activity via its effects on local conformation or conformational stability.

The 76 residues of ubiquitin represent a sequence uniquely conserved in evolution (Ozkaynak et al., 1984). This conservation presumably reflects the multiple components to which ubiquitin must be recognizable during its activation and conjugation to protein in the process of ATP-dependent proteolysis and in other ubiquitin-dependent events (Bush & Goldknopf, 1981; Hershko, 1986; Hershko & Ciechanover, 1986; St. John et al., 1986; Rechsteiner, 1987). The role of the carboxyl terminus of ubiquitin in its activation and subsequent conjugation of proteins has been characterized (Wilkinson & Audhya, 1981; Sobhanadity et al., 1988) and the importance of several other residues investigated by chemical modification and site-specific mutagenesis (Cox & Wilkinson, 1986; Ecker et al., 1987; Chau et al., 1989). Apart from Lys-48 and residues at or near the carboxyl terminus, however, no single residue strictly essential to ubiquitin activity has been localized.

Ubiquitin (Ub)[1] contains a single conserved methionine at position-1 (Schlesinger et al., 1975). Crystallographic analysis indicates that the methionine sulfur is hydrogen bonded to the backbone NH of Lys-63 (Vijay-Kumar et al., 1987). In a preliminary study, this laboratory reported that oxidation to the sulfone was without significant effect on ubiquitin biological activity, but was associated with an unusual low-pH conformational transition (Breslow et al., 1986a). Because this conformational change appeared to represent a molecular expansion unaccompanied by a significant change in α-helix or β-sheet content, it was suggested that ubiquitin contained two domains, the separation of which was facilitated by

[1] Abbreviations: Ub, ubiquitin; HPLC, high-performance liquid chromatography; CD, circular dichroism; NMR, nuclear magnetic resonance; TPCK, *N*-tosylphenylalanine chloromethyl ketone; TSP, sodium 3-(trimethylsilyl)propionate-d_4.

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