Initiation Codons in Mammalian Mitochondria: Differences in Genetic Code in the Organelle

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Received March 17, 1987; Revised Manuscript Received June 19, 1987

ABSTRACT: The bovine mitochondrial gene products ND2 and ND4, components of NADH dehydrogenase, have been purified from a chloroform/methanol extract of mitochondrial membranes, and the human mitochondrial gene products ND2 and cytochrome *b* have been obtained by similar procedures. They have been identified by comparison of their amino-terminal protein sequences with those predicted from DNA sequences of bovine and human mitochondrial DNA. All of the proteins have methionine as their amino-terminal residue. In bovine ND2, this residue is encoded by the "universal" isoleucine codon AUA, and the sequences of human cytochrome *b* and bovine ND2 demonstrate that AUA also encodes methionine in the elongation step of mitochondrial protein synthesis. In human ND2, the amino-terminal methionine is encoded by AUU, which, as in the "universal" genetic code, is also used as an isoleucine codon in elongation. Thus, AUU has a dual coding function which is dependent upon its context.

The DNA sequences of mammalian mitochondrial genomes revealed that mitochondria have a modified genetic code. In particular, comparison of the protein sequence of the bovine cytochrome oxidase subunit II (COII) with the sequence of the corresponding human gene indicated that UGA is used as a tryptophan codon in the mitochondrion and is not a termination codon (Barrell et al., 1979). This was confirmed when the bovine gene was sequenced (Young & Anderson, 1980), and the same change was found also in the yeast mitochondrion (Macino et al., 1979; Fox, 1979). Comparison of the protein sequence of bovine COII with its gene sequence also showed that AUA, a "universal" codon for isoleucine, codes for methionine during the elongation step of protein synthesis (Young & Anderson, 1980), and the presence of AUA codons in the human mitochondrial COII gene in some of the positions occupied by methionine in the sequence of the bovine protein suggested a similar difference in the human mitochondrial genetic code (Barrell et al., 1979). A number of other differences with the universal code were also proposed. These include the use of the codons AUA and AUU as initiation codons. They both encode isoleucine in the universal code but appear to be able to act as initiation codons in mitochondria probably specifying methionine in the case of AUA and possibly isoleucine in the case of AUU (Anderson et al., 1981, 1982). Until the present work, these assignments have been tentative and have not been tested because the appropriate protein products of the mitochondrial DNA had not been isolated and characterized. This is a reflection of the extreme hydrophobicities of these proteins coupled with a lack of suitable isolation procedures for hydrophobic proteins. Recently, we found that a group of hydrophobic proteins could be extracted from bovine mitochondrial membranes with organic solvents, and the extracts were fractionated by chromatography in a chloroform/methanol mixture, allowing us to isolate two hydrophobic membrane components of ATP synthase (Fearnley & Walker, 1986). These methods have now been extended further, and we have been able to purify for the first time the products of the bovine mitochondrial ND2 and ND4 genes, two hydrophobic components of NADH dehydrogenase (Chomyn et al., 1985), and also the human mitochondrial gene products ND2 and cytochrome *b*. By sequence analysis of these proteins, first, we have established that in bovine mitochondria AUA encodes methionine both in initiation and in elongation of protein synthesis. Second, we have shown that in the human mitochondrial ND2 gene, AUU codes for methionine in initiation of protein synthesis. Otherwise, as in the universal code, it is an elongation codon for isoleucine.

Materials and Methods

Mitochondria and Submitochondrial Particles. Mitochondria were isolated from bovine hearts (Smith, 1967), and submitochondrial particles were prepared from them according to Cattell et al. (1971). Mitochondria were isolated from human placentas by a similar method except that additional washes of both tissue and homogenate were incorporated in order to remove blood. The recovery of mitochondrial protein from a placenta (ca. 600 g) was 300 mg.

Extraction of Proteins. Mitochondria (300 mg) and submitochondrial particles were suspended in 50 mL of 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris·HCl), pH 7.0, and extracted with chloroform/methanol (1 L; 2:1 v/v) according to Fillingame (1976). The chloroform was washed with 10 mM Tris·HCl, pH 7.0, before use. Also, the addition of 2-mercaptoethanol (1 mM) to the chloroform/methanol to be used for extraction was found to reduce problems of aggregation and insolubility of proteolipids otherwise encountered during the extraction procedure (Fillingame, 1976). In order to extract the human ND2 protein, mitochondria were suspended in 50 mM Tris·HCl, pH 7.0, and extracted with chloroform/methanol (2:1 v/v) as above except that the chloroform was prewashed with 50 mM Tris·HCl, pH 7.0, before use. Human ND2 was not detected in the extracts made in the presence of 10 mM Tris·HCl buffer.

Protein Purification. Crude protein extracts dissolved in chloroform/methanol (2:1 v/v; ca. 2 mL) were applied to a column of Fractogel HW55(S) (190 cm × 2.0 cm i.d.) (Fearnley & Walker, 1986). Pure bovine ND2 protein and human cytochrome *b* were obtained by this procedure. Some of the fractions recovered from these size fractionations were desalted (Fillingame, 1976) and subjected to cation-exchange chromatography on a column 11.0 cm × 1.0 cm i.d. of

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0006-2960/87/0426-8247$01.50/0

Table I: Amino Acid Compositions of Some Proteins Isolated from Bovine and Human Mitochondria

	bovine				human			
	ND2		ND4		ND2		cytochrome *b*	
amino acid	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*
aspartic acid	21.0	18	29.2	28	21.8	20	28.5	26
threonine	38.4	38	32.0	35	39.6	43	32.2	30
serine	23.8	26	30.1	38	19.2	28	24.6	29
glutamic acid	15.7	14	21.1	19	15.7	16	10.8	12
proline	21.9	21	21.2	20	21.5	23	19.4	23
glycine	15.9	14	25.3	18	14.1	13	22.3	24
alanine	20.3	17	28.9	28	18.7	20	33.3	25
cysteine	n.d.[c]	0	n.d.	3	n.d.	0	n.d.	2
valine	13.3	13	16.7	18	8.4	8	13.2	10
methionine	40.6	43	24.1	36	22.6	25	19.4	15
isoleucine	25.1	35	34.1	41	30.7	31	34.3	39
leucine	51.4	53	87.4	92	63.1	64	65.8	64
tyrosine	9.0	8	13.2	17	10.6	10	15.2	17
phenylalanine	19.0	16	20.3	21	15.8	15	22.1	24
histidine	5.4	5	9.0	11	5.7	4	12.0	12
lysine	12.3	13	9.2	11	9.0	12	8.5	9
arginine	2.9	3	11	10	5.2	4	6.0	7
tryptophan	n.d.	10	n.d.	13	n.d.	11	n.d.	12
total residues		348		459		347		380

[a] By amino acid analysis of samples hydrolyzed for 24 h. [b] From gene sequence (Anderson et al., 1981, 1982). The values for aspartic acid are the sum of aspartate and asparagine, and those for glutamic acid are the sum of those for glutamate and glutamine. [c] n.d., not determined.

Fractogel CM-650(S) in chloroform/methanol mixtures. The proteins were applied in chloroform/methanol (2:1 v/v). Then the column was washed successively with chloroform/methanol [2:1 v/v (20 mL)], chloroform/methanol [1:1 v/v (20 mL)], and chloroform/methanol/water [5:5:1 v/v (20 mL)]. This was followed by a gradient of ammonium acetate from 0 to 150 mM in chloroform/methanol/water [5:5:1 v/v (Graf & Sebald, 1978)]. Human ND2 was freed from small amounts of cytochrome *b* by this procedure. ND2 eluted at 45 mM ammonium acetate (see Figure 1) whereas cytochrome *b* was not recovered. The purity of proteins was verified by polyacrylamide gel electrophoresis and by sequence analysis of individual fractions following a deformylation step.

Polyacrylamide Gel Electrophoresis. Fractions from chromatography experiments and purified proteins were examined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) (Laemmli, 1970) on gradient gels (10–30% polyacrylamide) and stained with PAGE blue 83 (BDH Biochemicals, Poole, U.K.).

Protein Chemical Methods. The methods used for amino acid analysis, removal of *N*-formyl groups, and protein sequence analysis have been described previously (Fearnley & Walker, 1986). Phenylthiohydantoin–amino acids were identified with an Applied Biosystems 120A liquid chromatograph "on-line" to the gas phase sequencer. Chromatographic data were collected with a Drew Scientific data capture unit and processed with the aid of the computer program CHROMAC 412 operated in a MacIntosh Plus computer.

Results

Purification of Proteins. The chloroform/methanol extract of bovine mitochondria contains approximately 13 proteins with apparent molecular weights ranging from approximately 4K to 50K. Chromatography of this extract on Fractogel HW55(S) resolved the three largest components. They have apparent molecular weight values of approximately 39K, 30K, and 18K, respectively (see Figure 1A). This separation is an improvement over that achieved with lipophilic Sephadex (LH-60) where components with M_r >10K are excluded from the matrix and are not resolved from each other (Fearnley & Walker, 1986). Amino acid compositions of the purified proteins were found to be similar to the predicted compositions of the mitochondrial gene products ND2, ND4 (see Table I), and ATPase 6 (Fearnley & Walker, 1986). In the case of ND2, the unusually high methionine content (43 out of 348 residues) was particularly characteristic. These identifications were confirmed by N-terminal protein sequence analysis (see below). ND2 and ND4, formerly known as URFs 2 and 4, respectively, (Anderson et al., 1982), have been identified by immunological studies as components of complex I or NADH dehydrogenase (Chomyn et al., 1985). However, until the present work, they had not been isolated and characterized.

The chloroform/methanol extract of human mitochondria contains proteins with a spectrum of molecular weights different from the bovine extract. Also, the composition of the extract is influenced by the concentration of the buffer used to resuspend the mitochondria. This was used to advantage in the purification of ND2 and cytochrome *b*. In the presence of 10 mM buffer, it was observed that one component with an apparent molecular weight of about 30K was extracted and on further analysis proved to be cytochrome *b*. When the extraction was performed in the presence of 50 mM buffer, an additional component was present in this size range (Figure 1B). It was not resolved from cytochrome *b* by size fractionation, but sequence analysis of the mixture indicated the presence of both cytochrome *b* and ND2. Pure ND2 was obtained from this mixture by ion-exchange chromatography on Fractogel CM-650(S) (see Figure 1C); cytochrome *b* was not recovered.

Characterization of Proteins. N-Terminal sequence analysis of the isolated proteins by automated Edman degradation required that the proteins were first treated with mild acid; otherwise, no phenylthiohydantoins were recovered. This is because of the presence in proteins encoded in mitochondrial DNA of an N-terminal formylmethionine residue (Smith & Marcker, 1968). The sequences determined in the four proteins (see Figure 2) show that they all have N-terminal methionine and so prove that both AUA and AUU can act as initiation codons specifying methionine. They confirm that AUA is an elongation codon for methionine and that AUU is an elongation codon for isoleucine.

The molecular weight values for the proteins calculated from the predicted protein sequences are considerably greater than those estimated by polyacrylamide gel electrophoresis in the

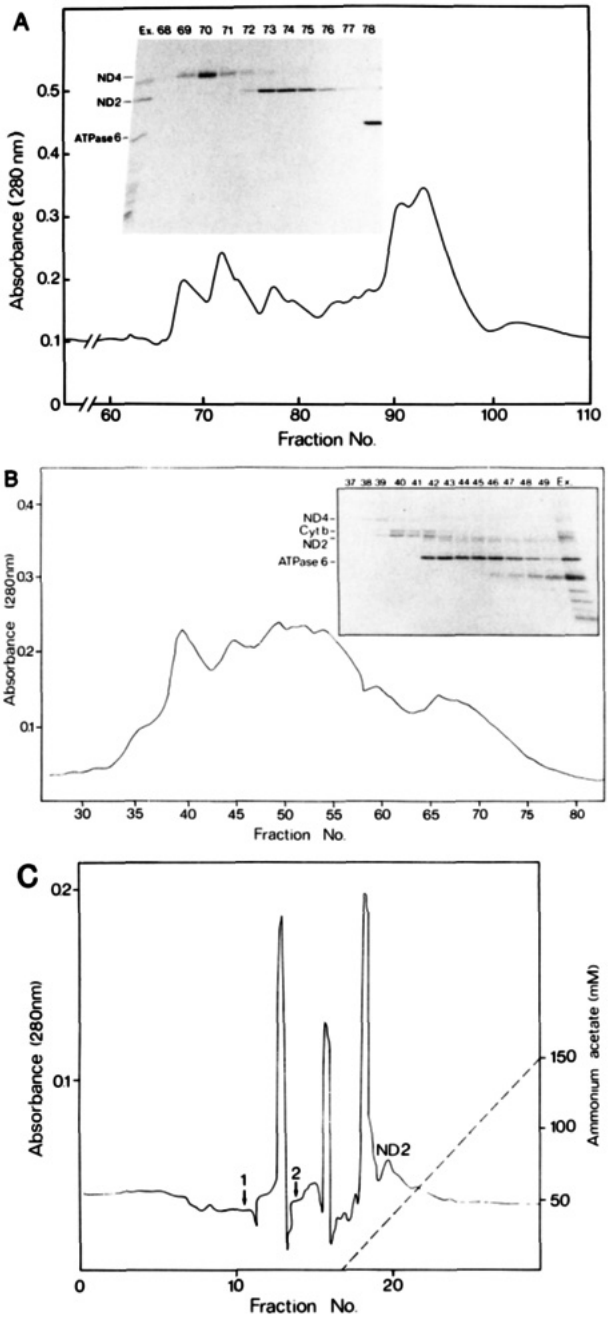

FIGURE 1: Purification of mitochondrial proteins. Fractionation on Fractogel HW55(S) of chloroform/methanol extract (Ex.) of (A) bovine submitochondrial particles and (B) human mitochondria extracted in the presence of 50 mM Tris·HCl. Polyacrylamide gel electrophoresis analysis of the extracts and fractions is shown in the insets. (C) Purification of human ND2 by ion-exchange chromatography on Fractogel CM-650(S). (1) Chloroform/methanol (1:1 v/v); (2) chloroform/methanol/water (5:5:1 v/v); (---) salt gradient.

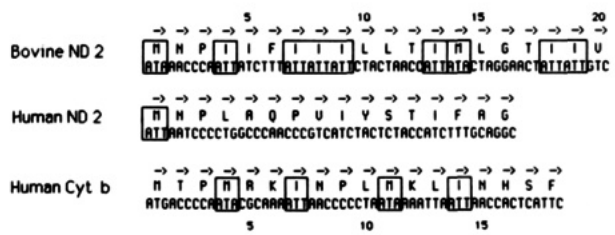

FIGURE 2: Amino-terminal protein sequences determined in bovine and human mitochondrial gene products. The arrows indicate the residues sequenced by Edman degradation of purified proteins after mild acid treatment to remove N-terminal formyl groups. The DNA sequences are from Anderson et al. (1981, 1982). Boxes indicate the positions of AUA and AUU codons.

Table II: Molecular Weights of Some Proteins Encoded in Mitochondrial DNA

protein	by gel electrophoresis	from sequence[a]
bovine ND2	30 100	39 283
bovine ND4	39 000	52 128
bovine ATPase 6	18 100	24 816
human ND2	30 500	38 989
human cytochrome *b*	33 000	42 758

[a] Includes N-terminal formyl group.

presence of SDS (see Table II). However, the underestimation by gel electrophoresis of the molecular weights of very hydrophobic proteins is a common occurrence, well-known examples being bacteriorhodopsin, bovine cytochrome *b*, and bovine cytochrome oxidase subunit I (COI).

Discussion

Mitochondrial Genetic Code. The experiments described above establish further differences between the universal genetic code and those used in human and bovine mitochondria. The N-terminal sequence of bovine ND2 shows that AUA can serve as an initiation codon specifying methionine. This is perhaps not unexpected as earlier it has been shown that in subunit II of cytochrome *c* oxidase (Young & Anderson, 1980) AUA specifies methionine in elongation, a finding confirmed in the present work. Moreover, a protein sequence analysis of human cytochrome *b* shows that the assignment of AUA as an elongation codon for methionine is also maintained in the human mitochondrial genetic code. In fungal mitochondria, the amino acid specified by AUA appears to be species dependent; it probably encodes methionine in *Saccharomyces cerevisiae* (Hudspeth et al., 1982) but isoleucine in *Aspergillus nidulans* (Waring et al., 1981) and *Neurospora crassa* (Browning & RajBhandary, 1982). It is probably an isoleucine codon also in maize mitochondria (Isaacs et al., 1985).

The sequence analyses of human cytochrome *b* and ND2 establish a dual role for the codon AUU. It is an isoleucine codon in elongation of cytochrome *b*, but it also specifies the initiator methionine residue of ND2. So this latter experiment removes the uncertainty of the amino acid specified by this particular codon (Anderson et al., 1981). In *Escherichia coli*, the codons GUG (Gold et al., 1981) and UUG (Young et al., 1981; Poulis et al., 1981) both have a dual role, acting either as initiation codons specifying methionine or as internal codons for valine and leucine, respectively.

AUU is not apparently employed as an initiation codon elsewhere in either bovine or human mitochondria, but its use as an initiation codon has also been proposed in the mouse ND1 gene (Bibb et al., 1981), in rat ND3 (Grosskopf & Feldman, 1982) and possibly ND1 (Saccone et al., 1981), and in five *Drosophila* mitochondrial genes, namely, those for A6L, ND2, ND3, ND5, and ND6 (Clary & Wolstenholme, 1985). As with the AUU initiation codon for human ND2, uncertainty also surrounded the assignment of these codons. In all cases, they were tentatively assigned as specifying isoleucine, but the possibility that the mouse AUU initiation codons could alternatively specify methionine was also considered (Bibb et al., 1981). It now seems likely that AUU in initiator positions will specify methionine in these other species. In two other mouse mitochondrial genes, those for ND3 and ND5, the universal isoleucine codon AUC has been advanced as the most probable initiation codon (Bibb et al., 1981); presumably, it too specifies methionine (see below). Another variation postulated for a mitochondrial initiation codon is that the gene for *Drosophila* COI starts with a four-base codon AUAA (de

Bruijn, 1983). All of these proposals concerning initiation of translation in mouse, rat, and *Drosophila* mitochondria are based solely upon interpretation of DNA sequences, and further experiments are required to confirm the assignments.

Mitochondrial Methionyl-tRNAs. Proteins synthesized in mitochondria, as in prokaryotes, have an N-terminal formylmethionine (Smith & Marcker, 1968; Galper & Darnell, 1969; Epler et al., 1970), and in yeast mitochondria, two isoaccepting methionyl-tRNAs have been characterized (Canaday et al., 1980; Sibler et al., 1985). Their sequences show that they are the products of two different genes, that they have 46 bases in common, and that both have a CAU anticodon. The $tRNA_m^{Met}$ must recognize both AUG and AUA, and the question arises of how this is done. Two examples are known of specific recognition of AUA by the CAU anticodon. They are the AUA-specific $tRNA^{Ile}$ of *E. coli* (Kuchino et al., 1980) and of bacteriophage T4 (Fukada & Abelson, 1980) which have modified C residues in the first position of the anticodon. However, the C residue of the CAU anticodon of yeast $tRNA_m^{Met}$ is unmodified, giving rise to the suggestion that the decoding properties of this tRNA are due to the presence of an extra unpaired nucleotide within the base-paired TΨC stem (Sibler et al., 1985).

The situation in mammalian mitochondria is unclear. Only one $tRNA^{Met}$ gene, coding for a tRNA with a CAU anticodon, is apparent in the complete mitochondrial DNA sequences (Anderson et al., 1981, 1982; Bibb et al., 1982). However, there is evidence in mammals that both $tRNA_f^{Met}$ and $tRNA_m^{Met}$ are specified by that single gene [discussed by van Etten et al. (1982)]. At least two hypotheses can be advanced in explanation. The first is that a gene for a second $tRNA^{Met}$ has not been detected in the mitochondrial DNA sequence because of unusual structural features that make it difficult to be recognized as a tRNA gene, as, for example, with $tRNA_{AGY}^{Ser}$ (de Bruijn et al., 1980). Another possibility is that there is only one mitochondrial $tRNA^{Met}$ gene and that specificity for elongation or initiation is conferred by specific modification of the tRNA. For example, modification of the C residue of the CAU anticodon could produce a $tRNA_f^{Met}$ able to recognize all four AUN codons as is required in mouse mitochondria. This proposal differs from the decoding of other four-codon families in mammalian mitochondria; this is achieved by U/N wobble in the first anticodon position (Anderson et al., 1981), the U probably being modified, as in *Neurospora* mitochondria (Heckman et al., 1980). The unmodified form of the mammalian $tRNA^{Met}$ could have some other structural feature (as in the yeast mitochondrion, for example) to enable it to function as a $tRNA_m^{Met}$ able to recognize both AUG and AUA. Other models can also be considered. For example, it may be that there is only one tRNA species present in mitochondria and that it is able to function both in initiation, recognizing all four AUN codons as methionine, and in elongation when only AUG and AUA are read as methionine. Indeed, only one $tRNA^{Met}$ has been characterized in both bovine and mosquito mitochondria: their anticodon stem is characteristic of a conventional initiator tRNA whereas the conformation of the anticodon loop appears to be characteristic of a conventional elongator tRNA (Dubin & HsuChen, 1984).

Registry No. NADH dehydrogenase, 9079-67-8; cytochrome *b*, 9035-37-4.

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Degradation and Deposition of Amyloid AA Fibrils Are Tissue Specific†

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Received March 11, 1987; Revised Manuscript Received June 5, 1987

ABSTRACT: The complete amino acid sequences of two related AA proteins (M_r 9700 and 5300) derived from thyroid tissue from a patient, NOR, with the autosomal recessive disease familial Mediterranean fever were determined. Heterogeneity found at position 52 indicates these proteins are fragments of two allelic or isotypic SAA precursor molecules similarly degraded at unusual sites and deposited in the thyroid. Degradation appears to be tissue and/or enzyme(s) specific since the carboxy terminus of both fragments is Ala-Ala and is different from other AA amyloid fibrils extracted from various tissues in different patients. Electron micrographic studies reveal these fragments retain the characteristics of native amyloid fibrils under physiological conditions even after exposure to dissociating agents.

Amyloidosis comprises a heterogeneous group of diseases characterized by the systemic or localized extracellular deposition of proteinaceous fibrillar material in various tissues and organs. These fibrils can be isolated by flotation at low ionic strength and are composed of low molecular weight subunits which tend to aggregate. Fibrils have a β-pleated sheet conformation by X-ray crystallography and assume a green birefringence when stained with Congo red and viewed under a polarizing microscope.

There are four major types of systemic amyloidosis. Primary amyloidosis is seen in association with multiple myeloma and other plasma cell dyscrasias. The major fibrillar protein, AL, is the amino-terminal variable region or the intact light chain of immunoglobulin light chains (Glenner et al., 1970). Chronic infectious and inflammatory disease, as well as familial Mediterranean fever, may be associated with secondary amyloidosis. Here, the fibril subunit protein, AA (Levin et al., 1972; Sletten & Husby, 1974), is believed to form as a result of the enzymatic cleavage of an acute-phase reactant, serum AA protein. Systemic amyloid may be associated with some familial amyloidotic polyneuropathy (FAP) syndromes all of which have an autosomal-dominant mode of inheritance (Costa et al., 1978; Pras et al., 1981; Skinner & Cohen, 1981). FAP types 1 and 2 and senile cardiomyopathy (Westermark et al., 1977; Gorevic et al., 1980a) have been shown to be due to fibrils composed of prealbumin.

Recently, a novel form of amyloidosis has been described in patients on long-term hemodialysis (Gejyo et al., 1985; Gorevic et al., 1985). Fibrils consist of polymers of normal intact β_2-microglobulin, clinically presenting as tumoral masses in bone or causing peripheral nerve entrapment. Other tissues, such as skin and blood vessels, may also be affected, indicating the systemic nature of the disease. Localized forms of amyloid restricted to the central nervous system include hereditary cerebral hemorrhage with amyloidosis (HCHWA) due to a variant of cystatin C (or γ trace) (Cohen et al., 1983; Ghiso et al., 1986) and the β-protein in cerebrovascular and senile plaque core amyloid in Alzheimer's disease and Down's syndrome with dementia (Glenner & Wong, 1984; Masters et al., 1985).

Amyloid A protein (AA) is a heterogeneous proteolytic cleavage fragment of a larger precursor. The complete sequences and the molecular weight determinations of a number of partially sequenced AA proteins showed that most of them are 76 residues long although proteins of different lengths [5–13.4 kilodaltons (kDa)] have been reported (Ein et al., 1972; Levin et al., 1972; Sletten et al., 1976; Isobe et al., 1977, 1980; Husby & Sletten, 1980; Lian et al., 1980; Moyner et al., 1980; Van Rijswijk, 1981). Whether this diversity reflects fragmentation during isolation or different sites of proteolytic cleavage of the precursor, SAA, is still unresolved. SAA is part of HDL_3 and circulates as a complex of approximately 180 000 daltons with α 1–2 electrophoretic mobility (Benditt et al., 1979). Upon denaturation a 12 000-dalton protein component with AA cross-reactivity is derived. SAA is the presumptive precursor of AA amyloid (Anders et al., 1975; Linke et al., 1975; Rosenthal et al., 1976).

There are two major and four minor isotypes of human SAA (Eriksen & Benditt, 1980; Bausserman et al., 1980). The amino acid sequence of SAA1 (Parmelee et al., 1982) has two allelic forms, α and β, with a double substitution of alanine for valine and valine for alanine at positions 52 and 57, respectively. Partial sequence analysis of SAA2 shows that it

† Supported in part by U.S. Public Health Service Research Grants AM 01431, AM 02594, and AG 05891.
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0006-2960/87/0426-8251$01.50/0