Primary structure of duck amyloid protein A
The form deposited in tissues may be identical to its serum precursor

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The amino acid sequence has been determined for the major protein that accumulates in amyloid fibrils in tissues of the Pekin duck. With the exception of 16 residues at the amino terminus, this 106-residue protein is homologous with human serum amyloid protein A (104-residue apoSAA), which is the putative precursor of the 76-residue protein that accumulates in human patients with amyloidosis. Duck serum is shown to contain a protein that is immunologically related and approximately equal in size (12 kDa) to the deposited form in ducks. These results indicate that proteolytic processing of the precursor is not a necessary step in the deposition of amyloid fibrils, at least in the duck.

Amino acid sequence; Amyloid protein A; Amyloidosis; SAA protein; Acute-phase protein; Lipoprotein

1. INTRODUCTION

The distinguishing feature of reactive, secondary amyloidosis in man and other species is the extracellular accumulation in various tissues of a 9 kDa protein termed amyloid protein A (AA) [1,2]. Its putative precursor in humans, the 12 kDa HDL-associated, acute-phase protein SAA, is 28 residues longer at the carboxyl-terminus [3]. Although it is generally assumed that proteolytic removal of the carboxyl-terminal segment is required for the deposition of protein AA as amyloid fibrils, Pekin ducks have been found to accumulate in their tissues a protein that exhibits size and partial sequence similarity [4,5] to the precursor form in human HDL particles and to proteins predicted from the cDNA sequences of murine SAA [6,7]. The present study completes the amino acid sequence determination of the major protein of Pekin duck amyloid fibrils, enabling comparisons with AA-related proteins from other species, and obviating the assumption that proteolytic digestion of the precursor is necessary for the deposition process.

2. MATERIALS AND METHODS

Duck liver tissue was generously supplied by Dr R.H. Rigdon (University of Texas Medical Branch, Galveston), who has described the spontaneous occurrence of amyloidosis in white Pekin ducks [8]. The amyloid protein was extracted and partially purified by procedures used for the isolation of human protein AA [9]. After removal of two high- Ms contaminants on a 2.5 × 115 cm Sephadex G-50 column in 9% HCOOH, the major component (12 kDa) was lyophilized.

The protein was cleaved at methionyl bonds with cyanogen bromide [10]. The largest fragment, M3, was subdigested with trypsin at arginyl residues,

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Abbreviations: AA protein, amyloid protein A; HDL, high density lipoprotein; HPLC, high-performance liquid chromatography; LDL, low density lipoprotein; VLDL, very low density lipoprotein; SAA protein, serum amyloid A precursor

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with *Staphylococcus aureus* V8 protease (Miles) at glutamyl residues [3], and with 70% formic acid (57°C, 18 h) at a single Asp–Pro bond.

Fragments generated with cyanogen bromide were separated on a Sephadex G-25 column (2.5 × 110 cm) in 9% formic acid [10]. Other peptides generated by subdigestion were purified by HPLC using TSK G3000SW and SynChropak RP-P columns with acetonitrile gradients in dilute aqueous trifluoroacetic acid [3].

Amino acid compositions were determined using a Dionex D550 amino acid analyzer following the manufacturer’s specifications or with a Waters Picotag system [11]. Sequences were analyzed with a Beckman 890B or 890M sequencer in the presence of polybrene [12] and each phenylthiohydantoin was identified in two different HPLC systems [13–15].

Serum with an elevated SAA level, as determined by radioimmunoassay [16], was obtained from a member of a locally maintained flock of Pekin ducks that, 17 months previously, had been injected with a homogenate of amyloidotic duck liver [4]. The serum was fractionated by sequential centrifugation at densities of 1.06, 1.13, and 1.21 g/ml. Flotation layers collected at each density correspond to VLDL + LDL, HDL2, and HDL3, respectively. These three layers and the bottom layer of density > 1.21 were dialyzed against 0.85% NaCl/0.01 M phosphate, pH 7.4, then examined by Western blot using rabbit antiserum to duck protein AA and 125I-staphylococcal protein A [17].

SEARCH and ALIGN programs [18] were used with the August 13, 1986 protein database files of the National Biomedical Research Foundation.

### Table 1

<table>
<thead>
<tr>
<th>Fragment: Residues:</th>
<th>Protein 1–106</th>
<th>M1 1–21</th>
<th>M2 22–28</th>
<th>M3 29–106</th>
<th>R3 100–106</th>
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<tr>
<td>Ala (A)</td>
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<td>3.1 (3)</td>
<td>1.1 (1)</td>
<td>13.1 (13)</td>
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<tr>
<td>Arg (R)</td>
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<td>2.0 (2)</td>
<td>1.9 (2)</td>
<td>11.0 (11)</td>
<td>1.0 (1)</td>
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<tr>
<td>Asx (D+N)</td>
<td>16.7 (10+7)</td>
<td>3.7 (3+1)</td>
<td>1.2 (1+0)</td>
<td>11.8 (6+6)</td>
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</tr>
<tr>
<td>Glx (E+Q)</td>
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<td></td>
<td></td>
<td>5.7 (4+2)</td>
<td></td>
</tr>
<tr>
<td>Gly (G)</td>
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<td>4.0 (4)</td>
<td></td>
<td>14.0 (13)</td>
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<tr>
<td>His (H)</td>
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<td></td>
<td></td>
<td>1.6 (2)</td>
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<tr>
<td>Ile (I)</td>
<td>1.9 (2)</td>
<td></td>
<td></td>
<td>1.9 (2)</td>
<td></td>
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<tr>
<td>Leu (L)</td>
<td>3.2 (3)</td>
<td>1.0 (1)</td>
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<td>1.0 (1)</td>
<td>1.0 (1)</td>
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<tr>
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<tr>
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<td>0.6 (1)</td>
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<tr>
<td>Phe (F)</td>
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<td>1.8 (2)</td>
<td></td>
<td>1.0 (1)</td>
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<tr>
<td>Pro (P)</td>
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<td></td>
<td>4.6 (5)</td>
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<tr>
<td>Ser&lt;sup&gt;b&lt;/sup&gt; (S)</td>
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<td></td>
<td>2.4 (2)</td>
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<tr>
<td>Thr&lt;sup&gt;b&lt;/sup&gt; (T)</td>
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<td>0.8 (1)</td>
<td>1.0 (1)</td>
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</tr>
<tr>
<td>Trp (W)</td>
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<td>N.D. (1)</td>
<td></td>
<td>N.D. (3)</td>
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<tr>
<td>Tyr (Y)</td>
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<td>2.7 (3)</td>
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<tr>
<td>Val (V)</td>
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<td></td>
<td>2.2 (2)</td>
<td></td>
</tr>
</tbody>
</table>

| Number of residues | (106) | (21) | (7) | (78) | (7) |

<sup>a</sup> Residues/molecule by amino acid analysis or (in parentheses) from the sequence. Hydrolysis for 20 h (115°C, 6 N HCl) with a crystal of phenol and 0.05% mercaptoethanol

<sup>b</sup> Ser increased by 10%, Thr by 5% to compensate for destruction by acid
3. RESULTS

The results of Edman degradation of the intact protein and eight peptides derived therefrom are summarized in fig. 1. Together these nine analyses provide a set of overlapping data that delineates the sequence of the 106-residue protein. The molecular mass calculated from the sequence is 11587 Da.

Fragmentation at the two methionyl residues (table 1) provided a fragment, M3 (residues 29–106), which, upon Edman degradation, yielded a sequence that overlapped the amino-terminal sequence of the intact protein and extended the structure to Ser-74. After cleavages at glutamyl or arginyl residues in M3, six peptides were isolated (E1–E3 and R1–R3, respectively). Edman degradation of these peptides extended the sequence to the carboxyl-terminal residue, Pro-106 (fig. 1).

The only tryptic peptide lacking a carboxyl-terminal arginine was R3; hence, it must be the carboxyl-terminal tryptic peptide of the protein. Confirmation of the carboxyl-terminal sequence was provided by the sequence of AC1, a peptide obtained by acid cleavage of M3 at an Asp–Pro bond. Both the intact protein and M3 were resistant to digestion by carboxypeptidase Y, possibly due to aggregation or to the high proline content of this region.

Coomassie staining after electrophoresis of the HDL₃ fraction from Pekin duck serum revealed two faint bands in the 12 kDa region (fig.2A), only one of which reacted with antibody to duck protein AA (fig.2B). The mobility of the immunopositive band was virtually identical to those of duck AA and of human apoSAA₁. Similarly located immunopositive bands were seen in the electrophoretic patterns of (VLDL + LDL), HDL₂, and the layer of greatest density (not shown); the latter also exhibited faintly immunopositive bands in association with dye-stained bands at approx. 30, 50 and 70 kDa.

4. DISCUSSION

The present sequence analysis of Pekin duck amyloid protein A completes the structure of the protein and shows it to be homologous throughout much of its length to human and murine precursor forms (fig.3). The earlier report of a partial sequence by Gorevic et al. [5] had suggested heterogeneities at residues 33, 34 and 63, but none of these was observed in the present analysis. Their tentative assignment of Ala-Arg or Ser-Arg to the C-terminus was based on carboxypeptidase digests, and is at variance with our sequence data. However, endopeptidase contamination is a common problem in such digests. Their estimate of a
molecular mass of 12000 Da (from SDS gels) is very close to the value of 11587 Da calculated from our sequence.

A search of the protein databank for homologous sequences revealed only amyloid A proteins and their precursors in other animal species. Alignment scores of 14.7 to 18.2 standard deviation units [18] indicate extensive similarity among these mammalian and duck proteins in spite of approx. 300 million years of divergence. As shown in fig.3, the regions of greatest identity are in the middle of the precursor forms (residues 37–68, using the numbering of the human protein). The small difference of three residues at the carboxyl-terminus could reflect a minor post-translational proteolytic digestion of the duck protein, but its DNA sequence would be necessary to clarify this point.

The amino-terminal 16 residues of the duck protein do not resemble their mammalian counterparts (alignment scores < 1) although the latter are quite similar to each other (fig.3). This region in murine AA corresponds to the second of four exons [7] and its lack of homology with the duck protein may suggest a unique or rapidly mutating exon encoding this region of the duck protein. A search of the sequence database detected no homologous segment.

The deposited form of human protein AA is 28 residues shorter at the carboxyl-terminus than the circulating form of the SAA protein [3]. A similar difference between deposited and circulating forms exists in the mouse [21]. Amyloid fibrils in monkey and mink contain homologous proteins with similar or shorter C-termini (fig.3), but the presumed precursor forms have not been studied. It is generally assumed that the deposition event and the proteolytic removal of the carboxyl-terminus from a circulating precursor are in some way interdependent. However, the present study weakens that hypothesis because the longer rather than the shorter form is deposited in the amyloid fibrils of the duck. Small amounts of SAA-like molecules have also been detected in human amyloid deposits [22]. Although the putative precursor in the duck has not been studied in detail, the evidence in fig.2 indicates that the circulating form resembles human SAA in mass and may well be identical with the deposited form in the duck.

The alignment in fig.3 shows that human amyloid fibrils contain protein cleaved between Ser-76 and Leu-77, although Moyner et al. [23] have reported a slightly longer (but tentative) structure from a patient with Waldenström's macroglobulinaemia. This site of cleavage is just C-terminal to an amphipathic helical region predicted to end at residue 74 [3]. It is not clear what protease degrades the amyloid precursor SAA to the fibrillar AA deposit in humans, monkey, mouse or mink. Nor is it possible to state whether the lack of proteolytic cleavage in the duck is due to the lack of a protease or to a difference in structure of the precursor itself, for ex-
Fig. 3. Comparison of Pekin duck liver amyloid protein A with serum precursors (SAA) in man and mouse, and with amyloid fibrillar deposits (AA) in monkey and mink. The number system corresponds to the human precursor [3]. Residues identical with the duck protein are boxed (circled residues are excluded from the box). Hyphens denote gaps to optimize homology; <Q symbolizes a pyrrolidone carboxyl residue. An asterisk indicates the C-terminus of the deposited fibrillar form in man [19,20]. Sequences have been determined on the protein unless noted otherwise.

- Isolated from HDL. A variant with Ala-52 and Val-57 was also seen [3]. Other variants have been reported in patients with familial Mediterranean fever [20] and with Waldenström's macroglobulinaemia [23].
- Derived from murine liver library cDNA sequence [6]. Two other variants, SAA2 and SAA3, have also been reported [6,7].
- Isolated from Macaca mulatta liver [10].
- Isolated from mink liver [24].

ample, at the unique Arg-77 (human numbering) in fig.3. Whatever the explanation, it is not necessary to invoke a proteolytic cleavage in the process of deposition of amyloid fibrils in the Pekin duck.

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REFERENCES