Sato, S., Nakazawa, K., Hon-nami, K., & Oshima, T. (1981) *Biochim. Biophys. Acta 668*, 277-289.
Tanaka, M., Haniu, M., Matsueda, G., Yasunobu, K. T., Himes, R. H., Akagi, J. M., Barnes, E. M., & Devanathan, T. (1971) *J. Biol. Chem. 246*, 3953-3960.
Travis, J., Newman, D. J., LeGall, J., & Peck, H. D., Jr. (1971) *Biochem. Biophys. Res. Commun. 45*, 452-458.
Tsunoda, J. N., Yasunobu, K. T., & Whitely, H. R. (1968) *J. Biol. Chem. 243*, 6262-6272.
Yang, S.-S., Ljungdahl, L. G., & LeGall, J. (1977) *J. Bacteriol. 130*, 1084-1090.
Yasunobu, K. T., & Tanaka, M. (1973) in *Iron-Sulfur Proteins* (Lovenberg, W., Ed.) Vol. 2, pp 27-130, Academic Press, New York.
Yoch, D. C., & Valentine, R. C. (1972) *J. Bacteriol. 110*, 1211-1213.
Zubieta, J. A., Mason, R., & Postgate, J. R. (1973) *Biochem. J. 133*, 851-854.

Amino Acid Sequence of Amyloid-Related Apoprotein ($apoSAA_1$) from Human High-Density Lipoprotein†

David C. Parmelee,‡ Koiti Titani, Lowell H. Ericsson, Nils Eriksen, Earl P. Benditt, and Kenneth A. Walsh*

ABSTRACT: The amino acid sequence of apoprotein SAA from human high-density lipoprotein is derived by analysis of peptides isolated from enzymatic digests. This 104-residue sequence is 28 amino acids longer than the amyloid protein AA that accumulates in tissues during certain inflammatory conditions. Two species of protein, differing from each other at only two loci, were recognized and characterized.

A type of amyloidosis which occurs in many chronic inflammatory diseases is caused by deposits of amyloid protein AA[1] in extracellular sites (Cohen, 1967; Benditt & Eriksen, 1971). Samples of this material isolated from human or various animal tissues have similar molecular masses (ca. 9000 daltons) and similar amino acid sequences of 76 residues (Hermodson et al., 1972; Levin et al., 1972; Sletten & Husby, 1974). Sera from normal and diseased individuals contain a 100-200-kilodalton component which reacts with antiserum prepared against the tissue amyloid protein AA (Husby et al., 1973; Husby & Natvig, 1974). This large serum AA related antigenic material (SAA) contains a protein of about 12 000 daltons with an amino-terminal amino acid sequence identical with that of amyloid protein AA. The SAA, like amyloid AA, often is found in association with a variety of pathological conditions (Rosenthal & Franklin, 1975), and the appearance of SAA in response to infection or inflammation identifies it as an acute-phase reactant (Selinger et al., 1980). Recently, SAA was shown to be present in serum high-density lipoproteins, and it was demonstrated that acid treatment released a 10-15-kilodalton component which is thought to be a precursor (apoSAA) to the tissue amyloid protein AA (Benditt & Eriksen, 1977). Subsequently, this apoSAA material was shown to be comprised of two major apoproteins ($apoSAA_1$ and $apoSAA_2$) that have very similar amino acid compositions (Eriksen & Benditt, 1980). Their amino acid sequences are identical in the first 30 residues, except that $apoSAA_2$ lacks the amino-terminal arginine residue that is found in $apoSAA_1$. These two major apoproteins were noted later by others, and four additional minor forms with very similar amino acid compositions were separated on DEAE-cellulose (Bausserman et al., 1980). These six SAA polymorphs were indistinguishable in cationic and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, they each had a carboxyl-terminal tyrosine residue, and preliminary experiments indicated that the charge heterogeneity did not appear to reflect differences in carbohydrate content (Bausserman et al., 1980). The molecular weight of each polymorph is approximately 11 500, indicating that they are each comprised of about 100 residues. These data suggest that a peptide of approximately 24 residues is cleaved from the carboxyl-terminal end of the apoSAA material when deposited in tissue as amyloid protein AA. Here we report the 104-residue sequence of human $apoSAA_1$, of which the amino-terminal 76 residues are identical with those of amyloid protein AA.

Materials and Methods

Amyloid-related apoprotein SAA_1 was isolated from the HDL_3 fraction of a pool of human sera with elevated AA immunoreactivity, as described by Eriksen & Benditt (1980) with certain modifications as follows. The HDL_3 fractions were dialyzed against 0.85% NaCl-0.01 M sodium phosphate, pH 7.4, concentrated 9-fold with an Amicon PM-10 membrane and passed through a Sephadex G-200 column in the same buffer at 4 °C. The fractions rich in AA-immunoreactive material were pooled, dialyzed against water, lyophilized, delipidated, fractionated on Sephadex G-100, dialyzed, and lyophilized as described. Subsequent chromatography on a DEAE-cellulose column (1.6 × 67 cm) employed a linear gradient (560 mL) from 0.02 to 0.09 M Tris-HCl in 7 M urea at pH 8. Two peaks of AA-immunoreactive material, designated $apoSAA_1$ and $apoSAA_2$, were separated, dialyzed against 0.5% acetic acid, and lyophilized. From 1400 mL of pooled sera, processed in five batches, the yields of $apoSAA_1$ and $apoSAA_2$ were 11 and 13 mg, respectively.

† From the Department of Biochemistry (L.H.E. and K.A.W.), the Howard Hughes Medical Institute Laboratory (D.C.P. and K.T.), and the Department of Pathology (N.E. and E.P.B.), University of Washington, Seattle, Washington 98195. *Received February 9, 1982.* This work was supported by grants from the National Institutes of Health (GM15731 and HL03174).

‡ Present address: Deutsches Krebsforschungszentrum, D6900 Heidelberg, West Germany.

[1] Abbreviations: AA, amyloid protein A; HPLC, high-performance liquid chromatography; SAA, serum AA related antigenic material; TFA, trifluoroacetic acid.

0006-2960/82/0421-3298$01.25/0

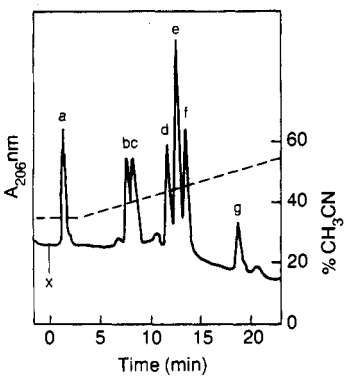

FIGURE 1: Chromatogram illustrating the final step in purifying apoSAA_1 (50 μg). A Waters μBondapak C_{18} column (0.39 × 30 cm) was initially equilibrated with a mixture of 65% solvent A (0.1% TFA) and 35% solvent B (acetonitrile containing 0.07% TFA). Gradient elution of the various proteins was performed at room temperature by using a flow rate of 2 mL/min and a full-scale detector response of 2.0 absorbance units. The various pools of fractions are alphabetically labeled. (---) Percent of acetonitrile. X indicates injection of the sample.

Lyophilized apoSAA_1 was dissolved in 6 M guanidine hydrochloride containing 0.02 M K_2HPO_4, pH 7, and separated into seven peaks by high-performance liquid chromatography using a column of Waters μBondapak C_{18} with an acetonitrile gradient in a Varian 5000 series instrument.

Lysine residues were modified by succinylation as described by Yaoi et al. (1964) except that the 0.2 M potassium phosphate (pH 8.0) contained 6 M guanidine hydrochloride (Heico) instead of 7 M urea. A 20-fold weight excess of succinic anhydride was added in small aliquots to a 1% protein solution in the above buffer at room temperature. The pH was maintained between 7 and 8 by using a pH stat. After 1 h the succinylated protein was desalted on a Sephadex G-25 column equilibrated with 0.1 M ammonium bicarbonate, pH 8.0, and lyophilized. Arginyl bonds were selectively cleaved with trypsin (TPCK treated, Worthington) by using a 1% solution of the succinylated apoSAA_1 in 0.2 M ammonium bicarbonate, pH 8.0, and a 2:100 molar ratio of enzyme to substrate. After digestion at 37 °C for 4 h the resulting peptides were lyophilized and separated by HPLC.

Cleavages with α-chymotrypsin (Worthington) and *Staphylococcus aureus* V8 protease (Miles) were done at pH 8 in 0.1 M ammonium bicarbonate at 37 °C for 6 h and at pH 7.8 in 0.05 M potassium phosphate at 37 °C for 18 h, respectively. All digests contained 1% protein and a 2:100 molar ratio of enzyme to substrate.

Tryptophanyl bonds were cleaved by a modification of the procedure of Omenn et al. (1970). Sixteen nanomoles of succinylated protein was dissolved in 0.8 mL of glacial acetic acid. BNPS-Skatole (Pierce) and 0.2 mL of water were then added, and the resulting solution was incubated for 6 h at room temperature in the dark. The mixture was diluted with an equal volume of water and the reagent extracted 3 times with 1 volume of 1-chlorobutane. The aqueous layer was lyophilized.

Selective cleavage of an aspartyl–prolyl bond was achieved in a peptide which was first blocked at the amino terminus in the sequenator cup by substituting an excess of phenyl isocyanate for phenyl isothiocyanate (Boosman, 1980). After the reagent was thoroughly extracted, the blocked peptide was cleaved by incubation in 70% formic acid (also in the cup) at 57 °C for 17 h. The formic acid was then evaporated under vacuum and the newly formed peptide beginning with proline was subjected to Edman degradations.

Amino acid compositions were determined with a Dionex D-500 amino acid analyzer by using the manufacturer's instructions. Suboptimal quantities of peptides were analyzed for compositions in several cases, in order to optimize the amount available for Edman degradation. Amino-terminal sequences were analyzed with a Beckman sequencer (Model 890C) according to the method of Edman & Begg (1967) as modified by Brauer et al. (1975). All analyses were performed in the presence of Polybrene (obtained from Pierce) (Tarr et al., 1978). Phenylthiohydantoin derivatives of amino acids were identified by two complementary systems of HPLC (Bridgen et al., 1976; Ericsson et al., 1977).

Results

Approximately 2 mg of amyloid-related apoprotein SAA_1 was isolated from the HDL_3 fraction of human serum and lyophilized. As shown in Figure 1, seven major peaks were observed during analysis by HPLC. Peak a is not retained by the column, lacked amino acids in a hydrolysate, and contains the guanidine of the sample solvent. Isoelectric focusing in polyacrylamide gels (data not shown) suggested that peaks b and c contained primarily apoprotein SAA_2 while peaks d–f contained apoSAA_1. Amino acid analyses supported this conclusion. Peak g contained material with a lower isoelectric point and an amino acid composition virtually identical with that of apolipoprotein A-II, a contaminant often found in amyloid preparations (Bausserman et al., 1980). The apoSAA_1 used in the subsequent analysis consisted of a lyophilized combination of pools d–f.

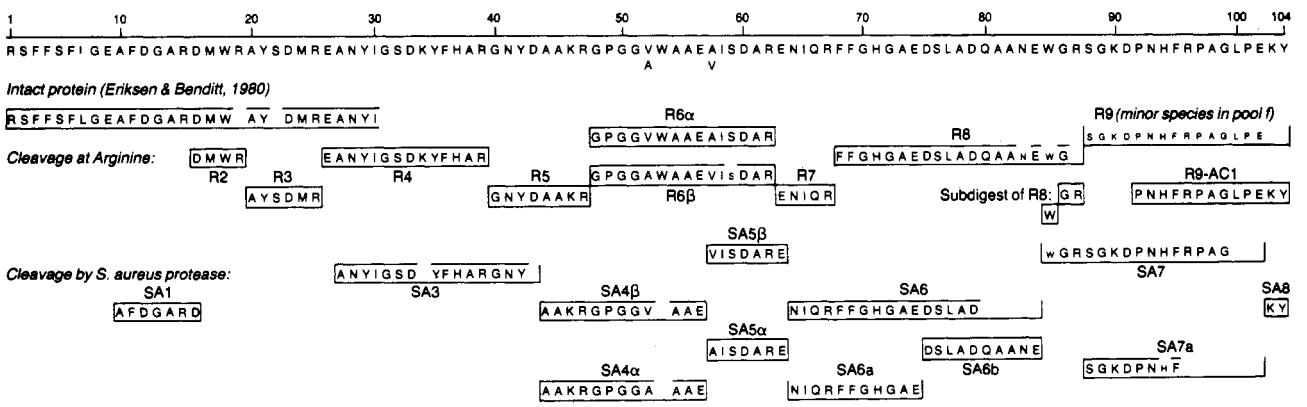

FIGURE 2: Summary of the proof of structure of apoSAA_1. Each bar represents a peptide with the enclosed sequence in one-letter code (Figure 5) as proven by Edman degradation (large letters) or as tentatively identified (small letters). Gaps in the upper enclosure of the bar indicate portions not clearly established. Prefixes R-, SA-, and AC- denote peptides derived by tryptic cleavage at arginyl bonds, digestion by *S. aureus* protease, and acid cleavage, respectively. Suffixes α and β identify peptides derived from an α or a β form of the protein.

Table I: Amino Acid Compositions[a] of Tryptic Peptides from Succinylated apoSAA$_1$[b]

peptide:	R0	R1	R2	R3	R4	R5	R6α	R6β	R7	R8	R9
source, pooled fractions in Figure 3A:	a	m	d	c[c]	e	c[c]	h	l	b	k[d]	g[e]
residue no.:	1	2–15	16–19	20–25	26–39	40–47	48–62	48–62	63–67	68–87	88–104
Asx		0.9 (1)	1.0 (1)	(1)	2.0 (2)	2.0 (2)	1.2 (1)	1.0 (1)	1.2 (1)	2.9 (3)	1.7 (2)
Thr											
Ser		1.0 (2)		(1)	1.1 (1)		1.0 (1)	(1)		0.3 (1)	0.4 (1)
Glx		0.7 (1)			1.4 (1)		2.0 (1)	1.2 (1)	2.0 (2)	3.3 (3)	1.6 (1)
Pro							(1)	0.7 (1)			3.5 (3)
Gly		1.5 (2)			1.4 (1)	1.1 (1)	1.7 (3)	2.3 (3)		2.7 (3)	1.5 (2)
Ala		1.7 (2)		(1)	2.2 (2)	2.0 (2)	4.2 (4)	4.1 (4)		3.3 (4)	0.7 (1)
Val							(1)	0.5 (1)			
Met			0.7 (1)	(1)							
Ile					1.0 (1)		1.0 (1)	1.0 (1)	0.9 (1)		
Leu		1.1 (1)								0.9 (1)	1.1 (1)
Tyr				(1)	1.8 (2)	0.7 (1)					0.6 (1)
Phe		4.0 (4)			1.2 (1)					2.3 (2)	1.2 (1)
His					0.7 (1)					0.8 (1)	0.5 (1)
Lys					0.9 (1)	1.0 (1)					1.9 (2)
Arg	1.0 (1)	1.0 (1)	0.9 (1)	(1)	1.0 (1)	1.0 (1)	1.0 (1)	1.2 (1)	1.0 (1)	0.5 (1)	1.0 (1)
Trp			(1)				(1)	(1)		(1)	
yield (nmol)	24	19	30	~8	10	25	8	8	24	18	16

[a] Residues per molecule by amino acid analysis or (in parentheses) from the sequence (Figure 2). [b] 45 nmol. [c] Fraction c was separated into peptides R3 and R5 by HPLC with a 12–16% gradient of acetonitrile. Fraction R3 was analyzed by sequenator only. [d] Ratio of R8 and R6β in fraction k is about 3/1. Data for R8 include this correction. [e] Corrected for 5 nmol of contaminating R4.

Two sets of overlapping peptides (Figure 2) were obtained by primary fragmentation with either trypsin (after succinylation) or staphylococcal protease. Peptides were isolated by HPLC and subjected to Edman degradation. When necessary, some of these peptides were subdigested to yield smaller peptides for additional analyses.

Peptides Derived by Cleavage at Arginine Residues. Forty-five nanomoles of succinylated protein was digested with trypsin, and the lyophilized mixture was separated by HPLC (Figure 3A). Seven peptides (R0, R1, R2, R4, R6α, R6β, and R7) were obtained (Table I) in pure form as judged by sequenator analysis. Two more (R3 and R5) were separated from fraction c (Figure 3A) by rechromatography in a less steep gradient. The amino acid compositions are given in Table I, and the sequenator analyses are summarized in Figure 2.

Tryptic peptide R4 was distributed among fractions e, f, and g in the proportions 10, 18, and 5 nmol, respectively. Only fraction e contained R4 as the single component as judged by both sequenator and amino acid analyses. Fraction f contained a small amount (ca. 4 nmol) of R9 as judged by amino acid analysis and by the observation of the minor sequence Ser-Gly-Lys-Asp-Pro-Asn-His-Phe-Arg-Pro-Ala-Gly-Leu-Pro-Glu. Fraction g contained principally R9 as judged by its composition, but Edman degradation revealed only a small amount of R4. Since the major portion of R9 (16 nmol) appeared to be in a blocked form in fraction g, and since its sequence was observed in fraction f to contain an Asp–Pro bond, fractions f and g were combined, blocked with phenyl isocyanate, and exposed to acidic conditions known to cleave this bond (see Materials and Methods). Subsequent Edman degradation of the mixture revealed a 13-residue sequence (R9-AC1 in Figure 2) which confirmed the trace sequence in fraction f beginning with Pro-Asn- and extending through Glu-Lys-Tyr-104.

Tryptic peptide R6 was also found in more than one peak, as judged by its Gly-Pro-Gly-Gly- amino terminus. Two of these fractions (h and i) contained a peptide R6α with the sequence Gly-Pro-Gly-Gly-Val-Trp-Ala-Ala-Glu-Ala-Ile-Ser-Asp-Ala-Arg; the other two (fractions k and l) contained a peptide R6β differing at the fifth and tenth residues where the alanine and valine were reversed. Apparently the preparation contains two products of closely related genes (or alleles). It is curious that each gene product is found in two separate peaks, but tryptophan-53 was only recognized during degradation of the more tightly retained fraction of each pair (peaks i and 1). It is probable that the tryptophan residues in fractions h and k had become modified (e.g., by oxidation) to an unrecognized form.

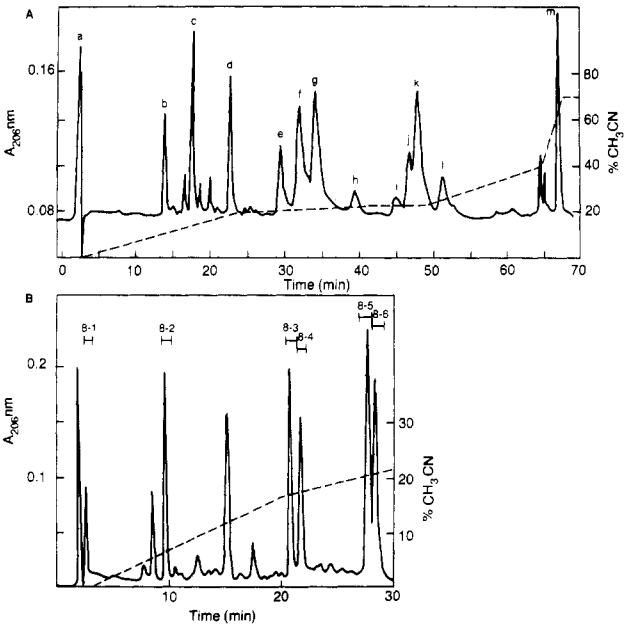

FIGURE 3: (A) Chromatogram illustrating the separation by HPLC of peptides formed by tryptic digestion of 0.5 mg of succinylated apoSAA$_1$. The chromatography was performed as described in Figure 1 except that the column was initially equilibrated with 0.1% TFA. The various pooled fractions are alphabetically labeled. (---) Percent acetonitrile. (B) Separation as above of peptides derived from fraction k (A) after successive digestions with chymotrypsin and with *S. aureus* protease (see the text).

Peptide R8 was also observed as a doublet peak (j and k). Possibly some interaction between R6β and R8 modifies the mobility of R8 in fraction k. Similar anomalous chromatographic behavior was seen above in the distribution of R4 among three peaks, two of which may result from interaction

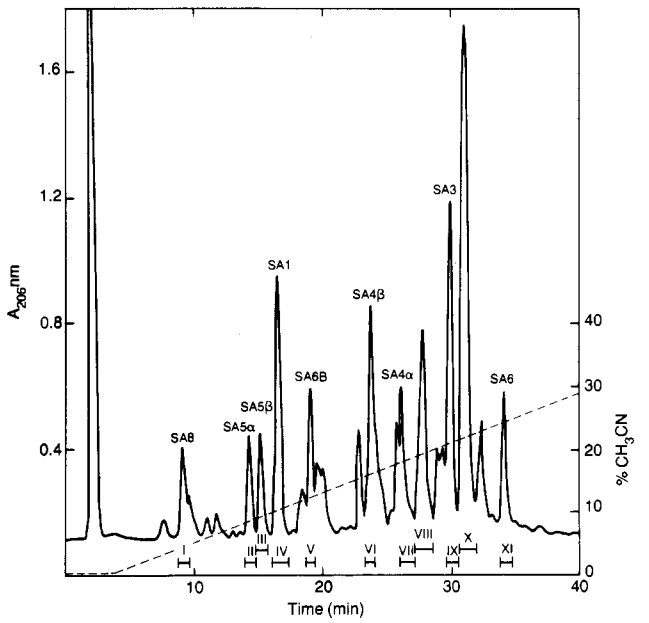

FIGURE 4: Separation by HPLC (as in Figure 3) of peptides derived by digestion of 0.55 mg of apoSAA$_1$ with *S. aureus* protease.

with R9 in its blocked and unblocked forms.

The carboxyl-terminal portion of R8 was obtained by subdigestion of fraction k with α-chymotrypsin, followed by *S. aureus* protease. This complex digest was fractionated by HPLC (Figure 3B), and six peptides were identified with R8. (Others are derived from an R6β contaminant.) Fraction 8-1 contained Gly-Arg, providing the carboxyl terminus of R8. Fraction 8-2 contained free tryptophan. This is assigned to residue 85 (Figure 2) on the basis of analyses of fractions 8-3 through 8-6, each of which had compositions consistent with residues 69 (or 70) through 84 (or 85). Whereas digestion with carboxypeptidase Y released from fraction 8-4 amino acids consistent with residues 84–77, similar digestion of fraction 8-5 released in addition an equivalent of tryptophan, placing it tentatively in position 85.

Peptides from Digestion with S. aureus Protease. A second set of peptides was obtained by cleavage of 60 nmol of protein with the protease from *S. aureus* and fractionation by HPLC (Figure 4). Ten pure peptides were obtained directly, and fraction VIII was further separated on the same column with a less steep gradient (12–16% acetonitrile) to yield pure SA7a and a peptide corresponding to residues 17–26 that was contaminated with SA7a. Fraction X was analyzed as a mixture, as discussed later.

Summary of Proof. The amino-terminal 42 residues of the protein are established by the published analysis of apoSAA$_1$ (Eriksen & Benditt, 1980) and its overlap with peptides R2, R3, R4, SA1, and SA3 (Figure 2). Peptide R5 extends the sequence to arginine-47. Residues 44–47 are clearly overlapped by both SA4α and SA4β, indicating that residue 52 is valine in a species α and alanine in a species β. Peptides R6α and R6β extend these sequences to residue 62, placing alanine at residue 57 of the α species and valine at residue 57 of the β species. The peptides SA5α and SA5β extend each species to glutamic acid-63. A single-residue overlap into R7 is sufficient because no other unplaced tryptic peptide has an amino-terminal glutamic acid. Peptides SA6 and SA6a provide overlaps to R8 that, together with SA6b, establish the sequence through glutamic acid-84.

Proof of the sequence of residues 84–92 was difficult. The subdigest of R8 (see above) provided the tentative carboxyl-terminus Glu-Trp-Gly-Arg (residues 84–87). The carboxyl

```
                    10                  20                  30
 1  R S F F S F L G E A F D G A R D M W R A Y S D M R E A N Y I
31  G S D K Y F H A R G N Y D A A K R G P G G V/A W A A E A/V I S D
61  A R E N I Q R F F G H G A E D S L A D Q A A N E W G R S G K
91  D P N H F R P A G L P E K Y  104
```

COMPOSITION

16 Ala A (15.4)	12 Gly G (12.4)	4 Pro P (3.9)
10 Arg R (10.2)	3 His H (2.9)	7 Ser S (5.8)
5 Asn N	3 Ile I (2.7)	0 Thr T (0.4)
9 Asp D (13.9)	3 Leu L (3.6)	3 Trp W (1.8)
0 Cys C (0)	4 Lys K (4.1)	5 Tyr Y (4.9)
2 Gln Q	2 Met M (1.9)	1 Val V (0.9)
7 Glu E (9.1)	8 Phe F (7.3)	

Mr = 11,685 No. of residues = 104

FIGURE 5: Amino acid sequence and composition of apoSAA$_1$ from human high-density lipoprotein. The one-letter code is identified in the lower portion. Two species, α and β, differ only at positions 52 and 57 where valine and alanine, respectively, are found in species α, whereas alanine and valine are found in species β. The amino acid composition (in parentheses) was reported by Bausserman et al. (1980) for the fraction designated SAA$_4$.

terminus of the protein (residues 88–104) is provided by the peptide R9, its internal sequence R9-AC1 and peptides SA7a and SA8. The overlap of R8 and R9 depends upon the peptide SA7 which was sequenced in a mixture with SA6a (in 1:2 ratio) obtained as fraction X in Figure 4. The results of this degradation were interpreted as follows. Although two residues were seen in each of the first 11 cycles of degradation, cycles 12–15 yielded a single sequence, Arg-Pro-Ala-Gly, which must be residues 96–99. After subtraction of residues 88–95 from the observed products in cycles 4–11, the remainder had the sequence Arg-Phe-Phe-Gly-His-Gly-Ala-Glu. This is clearly the sequence of residues 67–74, and the shorter contaminant peptide must be Asn-Ile-Gln-Arg-Phe-Phe-Gly-His-Gly-Ala-Glu (SA6a, Figure 2). The remaining residues identified in cycles 1–3 (Trp-Gly-Arg) must be derived from the longer peptide in the sequence, namely, Trp-Gly-Arg-Ser-Gly-Lys-Asp-Pro-Asn-His-Phe-Arg-Pro-Ala-Gly- (SA7 in Figure 2). The presence of tryptophan at residue 85 was confirmed by exposing 16 nmol of succinylated protein to cleavage at tryptophanyl bonds and then subjecting the whole mixture to seven cycles of Edman degradation. As expected, three sequences were seen, corresponding to bond cleavage at tryptophans-18, -53, and -85. The phenylthiohydantoins expected from residues 19–25 and 54–60 (including both alanine and valine at residue 57) were observed and in addition a third sequence corresponding to residues 86–92, Gly-Arg-Ser-Gly-Lys-Asp-Pro. Since these seven residues occur only in the fragment corresponding to residues 86–92, this experiment provided diagnostic confirmation of tryptophan at residue 85. The carboxyl terminus of R9, Lys-Tyr, is consistent with the observation of Bausserman et al. (1980) that the carboxyl terminus is tyrosine, preceded by lysine. However, their indications of phenylalanine at the third position do not agree with the present analysis.

The proposed sequence is in good agreement with both molecular mass determinations and the amino acid analyses (Figure 5) of Bausserman et al. (1980).

Discussion

Structural analyses of human apoSAA$_1$ were complicated by the minimal availability of material (ca. 2 mg) and the

Table II: Regions of Sequence Which Differ among apoSAA$_1$ and Its Amyloid Protein AA Counterpart

	residue no.				
	23	52–57	60	66	68–76
apoSAA$_1$					
present work	D[d]	V-W-A-A-E-A A-W-A-A-E-V	D	Q	F-F-G-H-G-A-E-D-S
amyloid AA					
Moyner et al. (1980)[a]	N	V W A R-A-A-E-A	D	E	F-F-G-H-G-A-E-N-S
Levin et al. (1972)[b]	D	A-R-A-A-E-V	N	Q	L-T-G-R-G-A-E-D-S
Sletten & Husby (1974)[c]	N	V-W-A-A-E-A	D	Q	F-F-G-H-G-A-E-N-S

[a] From the liver of a patient with Waldenström's macroglobulinaemia and amyloidosis. [b] From a patient with familial Mediterranean fever. [c] From the liver of a patient with chronic rheumatic disease. [d] One-letter codes are given in Figure 5.

presence of two polymorphic forms in the preparation. Most of the material was used for Edman degradation of the protein and fragments thereof, and little was used to establish good amino acid compositions. The two polymorphs were analyzed as a mixture, but the peptides isolated clearly establish that only two forms exist in this preparation. It is clear that other apoSAA forms exist (Bausserman et al., 1980), but detailed sequence data are not available to distinguish them from the α and β forms of apoSAA$_1$ defined in the present study.

The analysis itself is provided largely by two overlapping sets of peptides. Although the *S. aureus* protease exhibited the expected primary specificity toward glutamyl bonds, one arginyl and two aspartyl bonds were cleaved in significant yield. In two cases, a mixture of two peptides was subjected to Edman degradation without prior purification. As a result, the proof of sequence of residues 85–87 is not ideal, but the several analyses described are all consistent with the proposed sequence.

It is not clear whether the two forms of the protein are allotypes or isozymes. The described difference between the two forms is a curious double substitution resulting in identical compositions. In the corresponding region of sequence (residues 52–57), amyloid proteins AA from patients with various disorders have been observed with each form and with mixtures of both (Table II).

There is little doubt that the deposited form, amyloid protein AA, is a foreshortened version of the circulatory form, apoSAA$_1$. Decisive comparisons must allow for differences between reports of the sequence of human amyloid AA as summarized in Table II. Most of these differences are found in amide assignments. The present data were obtained by HPLC where errors are unlikely. It is possible that amides may be selectively lost during isolation of the proteins or that the various diseased tissue sources contain protein with actual differences in amide placement. The difference at residues 69–71 between Levin et al. (1972), Leu-Thr-Gly-Arg, and three other analyses including the present one, Phe-Phe-Gly-His, appears to be more significant and could reflect a specific inherited lesion in the protein derived from the patient with familial Mediterranean fever. It is interesting that the analogous protein in the monkey is clearly homologous to the human protein and has elements of both sequences in this region, namely, Leu-Leu-Gly-His (Hermodson et al., 1972).

Examination of the sequence of apoSAA$_1$ for patterns of hydrophobicity reveals that residues 1–24 and 50–74 would readily form amphipathic α helices of about seven turns each. Segrest et al. (1976) have already noted this feature in the first segment of amyloid AA. This character may well underlie the association of apoSAA$_1$ with the HDL particle, leaving two segments (residues 25–49 and 75–104) with unspecified function. It is of interest that the portion which is missing in the deposited AA form lacks the carboxyl-terminal segment and that the putative bond cleavage site is only two residues beyond the suggested amphipathic helix.

Amyloid protein AA consists of residues 1–76 of apoSAA$_1$, but it is not clear what protease cleaves the Ser–Leu bond. Moyner et al. (1980) obtained from a patient with macroglobulinaemia a somewhat longer form of protein AA, but the carboxyl terminus was not established.

At this time it is not clear what processes stimulate the production of apoSAA in liver (Selinger et al., 1980), how this protein finds its way to association with the HDL particle, or how prolonged inflammatory conditions lead to degradation of this protein and its deposition as amyloid fibrils. Knowledge of the detailed structure of the circulatory form may facilitate elucidation of these processes.

Acknowledgments

We are indebted to Roger D. Wade, Santosh Kumar, and Richard Granberg for technical assistance.

References

Bausserman, L. L., Herbert, P. N., & McAdams, K. P. W. J. (1980) *J. Exp. Med. 152*, 641.

Benditt, E. P., & Eriksen, N. (1971) *Am. J. Pathol. 65*, 231.

Benditt, E. P., & Eriksen, N. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4025.

Boosman, A. (1980) in *Methods in Peptide and Protein Sequence Analysis* (Birr, C., Ed.) p 513, Elsevier/North-Holland Press, New York.

Brauer, A. W., Margolies, M. N., & Haber, E. (1975) *Biochemistry 14*, 3029.

Bridgen, P. J., Cross, G. A. M., & Bridgen, J. (1976) *Nature (London) 263*, 613.

Cohen, A. S. (1967) *N. Engl. J. Med. 277*, 522.

Edman, P., & Begg, G. (1967) *Eur. J. Biochem. 1*, 80.

Ericsson, L. H., Wade, R. D., Gagnon, J., McDonald, R. M., Granberg, R. R., & Walsh, K. A. (1977) in *Solid Phase Methods in Protein Sequence Analysis* (Previero, A., & Coletti-Previero, M. A., Eds.) p 137, Elsevier/North-Holland Press, New York.

Eriksen, N., & Benditt, E. P. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 6860.

Hermodson, M. A., Kuhn, R. W., Walsh, K. A., Neurath, H., Eriksen, N., & Benditt, E. P. (1972) *Biochemistry 11*, 2934.

Husby, G., & Natvig, J. B. (1974) *J. Clin. Invest. 53*, 1054.

Husby, G., Natvig, J. B., Michaelsen, T. E., Sletten, K., & Höst, H. (1973) *Nature (London) 244*, 362.

Levin, M., Franklin, E. C., Frangione, B., & Pras, M. (1972) *J. Clin. Invest. 51*, 2773.

Moyner, K., Sletten, K., Husby, G., & Natvig, J. B. (1980) *Scand. J. Immunol. 11*, 549.

Omenn, G. S., Fontana, A., & Anfinsen, C. B. (1970) *J. Biol. Chem. 245*, 1895.
Rosenthal, C. J., & Franklin, E. C. (1975) *J. Clin. Invest. 55*, 746.
Segrest, J. P., Pownall, H. J., Jackson, R. L., Glenner, G. G., & Pollock, P. S. (1976) *Biochemistry 15*, 3187.
Selinger, M. J., McAdam, K. P. W. J., Kaplan, M. M., Sipes, J. D., Vogel, S. N., & Rosenstreich, D. L. (1980) *Nature (London) 285*, 498.
Sletten, K., & Husby, G. (1974) *Eur. J. Biochem. 41*, 117.
Tarr, G. E., Beecher, J. F., Bell, M., & McKean, D. J. (1978) *Anal. Biochem. 84*, 622.
Yaoi, J., Titani, K., & Narita, K. (1964) *J. Biochem. (Tokyo) 56*, 222.

Autophosphorylation of Rhodopsin Kinase from Retinal Rod Outer Segments†

Rehwa H. Lee,* Bruce M. Brown, and Richard N. Lolley

ABSTRACT: Rhodopsin kinase has been identified as a 68K protein that is more readily extracted from dark-adapted rod outer segments (dark-extract) than from illuminated rod outer segments (light-extract). We observed that a 68K protein is phosphorylated by endogenous protein kinase of dark- or light-extract of bovine rod outer segments and that the amount of incorporated radioactivity (^{32}P) was greater in the dark- than in the light-extract. Phosphorylation of the 68K protein is neither stimulated by cyclic nucleotides nor affected by the light or dark conditions of the phosphorylation reaction. Light- and dark-extracts were centrifuged simultaneously on individual sucrose density gradients revealing that the 68K phosphoprotein cosediments with endogenous rhodopsin kinase activity and that both greater ^{32}P incorporation and higher rhodopsin kinase activity are found in dark-extract as compared to light-extract. These findings suggest strongly that the 68K phosphoprotein and rhodopsin kinase are identical and that rhodopsin kinase undergoes autophosphorylation.

Protein phosphorylation is an important mechanism by which cellular metabolism or function is regulated (Greengard, 1978). It participates in the control of carbohydrate and lipid metabolism as well as synaptic transmission and muscle contraction (Kebabian, 1977; Nimmo & Cohen, 1977). On the molecular level, the phosphorylation of enzymes is known to alter the conformation of enzymes and, thereby, modulate their activity or kinetic characteristics (Krebs & Beavo, 1979).

In rod photoreceptors of bovine retina, phosphorylation of two endogenous proteins has been described. The phosphorylation of a 33K soluble protein is catalyzed by a cyclic nucleotide dependent protein kinase (CNPK)[1] which is present in the rod outer segment (ROS) (Lolley et al., 1977). The activity of CNPK is modulated by light-induced changes in cGMP concentrations which are high in the dark and low in the light (Woodruff & Bownds, 1979; Farber et al., 1978; Yee & Liebman, 1978; Fletcher & Chader, 1976).

Phosphorylation of the ROS membrane protein, rhodopsin, is catalyzed by a rhodopsin kinase (RK), the activity of which is affected by neither cyclic nucleotides nor light (Frank & Buzney, 1975; Weller et al., 1975). The RK phosphorylates only freshly bleached rhodopsin, but not dark-adapted rhodopsin or commonly used protein kinase substrates such as histone, protamine, or casein (Lee et al., 1981; Shichi & Somers, 1978). This enzyme is a 68K protein which exists in the cytoplasmic compartment of ROS in the dark and binds to the ROS membranes in the light (Kühn, 1978). It is suggested that light stimulates phosphorylation of rhodopsin by inducing conformational changes of the visual pigment, thereby providing RK with an appropriate binding site on the ROS membranes as well as a suitable substrate for phosphorylation.

In this paper, we report the phosphorylation of a 68K ROS protein by an endogenous ROS protein kinase. On the basis of (a) the molecular weight (68K) of this endogenous substrate, (b) its light-dependent affinity for ROS membranes, (c) its cosedimentation with RK activity during centrifugation, and (d) the absence of effects by light or cyclic nucleotides on its phosphorylation, we propose that this 68K phosphoprotein is phosphorylated rhodopsin kinase and that the enzyme undergoes autophosphorylation.

Experimental Procedures

Materials

Dark-adapted bovine retinal ROS, protein kinase substrate (PKS) from rat intestine, and alum-treated ROS membranes were prepared as described previously (Lee et al., 1981). [γ-^{32}P]ATP was purchased from New England Nuclear (2.5–4 Ci/μmol). Cronex X-ray film was from Du Pont.

Methods

Preparation of Light- or Dark-Extract from Bovine ROS. Peripheral proteins were extracted from light- or dark-adapted ROS membranes by the procedure of Kühn (1978). Briefly, a ROS pellet, which was prepared from six dark-adapted bovine retinas and kept in darkness at −70 °C until use, was homogenized in the dark in 1 mL of 20 mM Tris-HCl, pH

† From the Department of Anatomy, University of California at Los Angeles School of Medicine, Los Angeles, California 90024, and the Developmental Neurology Laboratory, Sepulveda Veterans Administration Medical Center, Sepulveda, California 91343. *Received September 17, 1981.* This work was supported by National Science Foundation Grant BNS 79-26806, National Institutes of Health Grant EY00395, and the Medical Research Service of the Veterans Administration.

* Address correspondence to this author at the Developmental Neurology Laboratory (151B9), Sepulveda Veterans Administration Medical Center.

[1] Abbreviations: ROS, rod outer segment(s); cAMP, adenosine cyclic 3′,5′-phosphate; cGMP, guanosine cyclic 3′,5′-phosphate; CNPK, cyclic nucleotide dependent protein kinase; PKS, protein kinase substrate; RK, rhodopsin kinase; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

0006-2960/82/0421-3303$01.25/0