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Complete Amino Acid Sequence of the 4Fe–4S, Thermostable Ferredoxin from *Clostridium thermoaceticum*†

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ABSTRACT: The complete amino acid sequence of the 4Fe–4S ferredoxin from the thermophilic bacterium *Clostridium thermoaceticum* has been determined. The protein is extremely thermostable and is the only known clostridial ferredoxin to contain a single [4Fe–4S] cluster. The sequence totals 63 residues and includes the first tryptophan (Trp-26) reported for a clostridial ferredoxin, and other amino acids not commonly found in clostridial or clostridial-like ferredoxins: methionine (Met-1), histidine (His-33), arginine (Arg-49), and leucine (Leu-9, -19, and -31). Sequence homology to clostridial and other 8Fe–8S ferredoxins is limited to eight to nine residues at the amino-terminal sulfhydryl grouping (Cys-10, -13, -16, and -20) and two to five residues in the carboxy-terminal region. This ferredoxin is, thus, sequentially distinct from all known clostridial ferredoxins and from other bacterial ferredoxins in both the 8Fe–8S and 4Fe–4S classes.

Yang et al. (1977) isolated an unusual ferredoxin from the homoacetate-fermenting, thermophilic, anaerobic bacterium *Clostridium thermoaceticum*. In addition to its high thermostability (60 min at 80 °C), iron and sulfur analyses and extrusion studies established only a single [4Fe–4S] cluster present per molecule of protein. This was in direct opposition to the previously coined term "clostridial ferredoxin" which was used to denote a ferredoxin with two [4Fe–4S] clusters. Moreover, the *C. thermoaceticum* ferredoxin contained 63 residues, rather than the 54–56 residues consistently reported for clostridial and related ferredoxins, and included a unique tryptophan residue, an amino acid not previously observed in a clostridial ferredoxin.

Several nonclostridial ferredoxins are now known to contain a single, low-potential [4Fe–4S] cluster: *Bacillus polymyxa* (Yoch & Valentine, 1972), *Bacillus stearothermophilus* (Mullinger et al., 1975), *Desulfovibrio gigas* (LeGall & Dragoni, 1966), *Desulfovibrio desulfuricans* (Zubieta et al., 1973), and *Spirochaeta stenostrepta* (Johnson & Canale-Parola, 1973). Thus, the sequence of the thermostable ferredoxin from *C. thermoaceticum* is of interest for comparison to the ferredoxins from thermophilic and mesophilic clostridia, as well as to the 4Fe–4S ferredoxins from other bacterial systems.

Materials and Methods

C. thermoaceticum was grown at 58 °C according to Yang et al. (1977). Ferredoxin was isolated as described previously (Yang et al., 1977) in yields of 7–9.5 mg of protein/310 g of cell paste.

Iodo[2-^{14}C]acetic acid (40–60 mCi/mmol) was obtained from Amersham, Arlington Heights, IL. Porcine trypsin and yeast carboxypeptidase Y came from Sigma Chemical Co., St. Louis, MO. Hydrazine was purchased from Fisher Sci-

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entific Co., Fair Lawn, NJ. All other chemicals were reagent grade and used without further purification.

Radioactive Labeling of Cysteine Residues. The cysteine residues of ferredoxin from *C. thermoaceticum* were labeled by using iodo[2-^{14}C]acetic acid, 2.5 μCi/mg of protein, essentially by the procedure of Crestfield et al. (1963). Guanidine hydrochloride, 6 M, was used as a denaturant, and then the reduced, anaerobic protein was reacted for 15 min with labeled iodoacetic acid followed by cold iodoacetic acid. Excess reagent was removed by chromatography on a foil-covered Sephadex G-25 (Pharmacia) column equilibrated with 0.6% NH_4OH.

Digestion of Ferredoxin with Trypsin. Tryptic digestion was done on 18 mg of S-carboxymethylated ferredoxin (CM-Fd) in 0.2 M NH_4HCO_3, pH 7.8. Two milligrams of porcine trypsin was added and allowed to react for 4 h at 37 °C. The sample was then applied to a Sephadex G-50 column (1.5 × 150 cm) equilibrated with the reaction buffer. The eluant was monitored by A_{220}, the peaks of absorbing material were pooled, and the samples were lyophilized repeatedly.

Carboxy-Terminal Analysis of Ferredoxin. Digestion with carboxypeptidase Y was performed according to Hayashi (1976) in 0.1 M pyridineacetate, pH 5.5 and 7.0, with and without 6 M urea. Samples containing 20 nmol (140 μg) of ferredoxin in 0.2 mL of buffer were digested with 14 μg of carboxypeptidase Y. An internal standard of 20 nmol of norleucine was included with each sample. Digestions were for 10 min to 18 h at 25 and 37 °C.

Samples for hydrazinolysis were treated with carboxypeptidase Y as described above for 2 h at 37 °C in pyridineacetate, pH 7.0, to give quantitative release of valine. The sample was lyophilized and placed in a vacuum desiccator over P_2O_5 for 2–3 days to obtain complete dryness. Hydrazinolysis was performed for 48 h at 80 °C as described by Travis et al. (1971) using dried argon and freshly opened hydrazine to avoid moisture contamination.

Automatic Sequencing. The amino-terminal sequences of ^{14}C-labeled S-carboxymethylated ferredoxin and tryptic peptides were performed by using a Beckman Model 890C sequencer. A 0.1 M Quadrol program (manufacturer no. 030176) was used for all analyses, and 3 mg of polybrene was typically added to the sequencing cup. A lysine-containing, tryptic peptide was also sequenced by using a Sequemat Mini-15 solid-phase sequencer. The peptide was coupled to diisothiocyanate glass, prepared according to Karr & Foster (1981), and subjected to automated Edman degradation as described by Laursen (1971). Residues from both sequencing systems were converted from the 2-anilino-2-thiazolone derivatives to phenylthiohydantoins and analyzed by high-pressure liquid chromatography (HPLC) (Travis et al., 1971), or back-hydrolyzed to free amino acids and analyzed by using a Beckman Model 119CL amino acid analyzer (Mendez & Lai, 1975).

HPLC separation was conclusive for the stable derivatives present except for the following unresolved pairs: Phe-Ile and Val-Met. These pairs were easily distinguished by amino acid analysis of a portion of the samples. The derivatives of Ser and Thr were destroyed during conversion to phenylthiohydantoins and not observed by HPLC analysis. They were determined by amino acid analysis following back-hydrolysis as Ala and α-aminobutyric acid, respectively. Alanine residues were, therefore, observed as Ala on both HPLC and amino acid analysis, while Ser appeared as a blank on HPLC and as Ala, using amino acid analysis. Tryptophan was corroborated by amino acid analysis as Gly/Ala (2:1). Cysteine residues were identified by scintillation counting of 5% of the ethyl acetate extract obtained during conversion and by amino acid analysis as Gly/Ala (1:1). Since acidic conditions are used in the conversion process, deamidation of the amide residues will occur to some extent. Therefore, Gln and Asn were considered the original residue if even a low percentage of either Gln or Asn was observed by HPLC analysis (Chin et al., 1981).

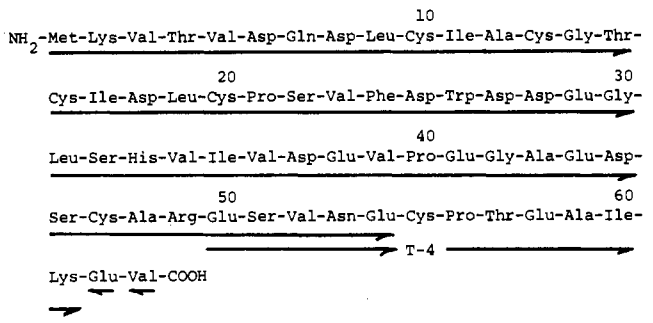

FIGURE 1: Complete amino acid sequence of the 4Fe–4S ferredoxin from *C. thermoaceticum*. The amino-terminal sequence was obtained from ^{14}C-labeled S-carboxymethylated whole protein, residues 1–54. Peptide T-4 was obtained from a tryptic digest. The carboxy-terminal and penultimate residues were determined by carboxypeptidase Y digestion and by carboxypeptidase Y digestion followed by hydrazinolysis (see Materials and Methods).

Table I: Amino Acid Composition of the Ferredoxin from *C. thermoaceticum* and of Peptide T-4 from Tryptic Digestion

amino acid	ferredoxin[a]	T-4
Asp	9.12 (9)	1.31 (1)
Thr	3.18 (3)	0.96 (1)
Ser	4.26 (4)	1.24 (1)
Glu	9.32 (9)	2.29 (3)
Pro	3.37 (3)	0.81 (1)
Gly	3.46 (3)	0.39 (0)
Ala	4.43 (4)	1.10 (1)
Val	7.83 (8)	1.29 (1)
Met	1.13 (1)	0.00 (0)
Ile	3.61 (4)	1.00 (1)
Leu	3.02 (3)	0.28 (0)
Phe	0.91 (1)	0.10 (0)
Tyr	0.06 (0)	0.05 (0)
His	0.86 (1)	0.09 (0)
Lys	1.83 (2)	0.85 (1)
Arg	1.04 (1)	0.04 (0)
Trp[b]	1.12 (1)	0.00 (0)
CM-Cys	5.91 (6)	1.21 (1)
total	63	12

[a] The values presented are the average of five analyses. Numbers in parentheses indicate the residues found by sequencing. [b] Determined from absorption of the S-carboxymethylated protein (ϵ_{280} = 5600 M^{-1} cm^{-1}) and by hydrolysis in the presence of 4% thioglycolic acid.

Results

The major portion of the ferredoxin from *C. thermoaceticum* was sequenced from the intact, ^{14}C-labeled S-carboxymethylated protein. Using 250 nmol of protein, initially, ferredoxin was sequenced from residues 1–46 and 1–54 on two samples (see Figure 1 and Table I). Repetitive yields were 94–96% based on Val at residues 5, 23, and 36. Initial yields were 55–65% of the added protein. This sequence included the single arginine residue (Arg-49) and established a definitive overlap sequence with the lysine-containing, tryptic peptide (residues 50–61) described below.

Tryptic digestion of ferredoxin yielded five peaks of material absorbing at 220 nm when chromatographed on Sephadex G-50. Peak 1 was an arginine-containing peptide (apparently,

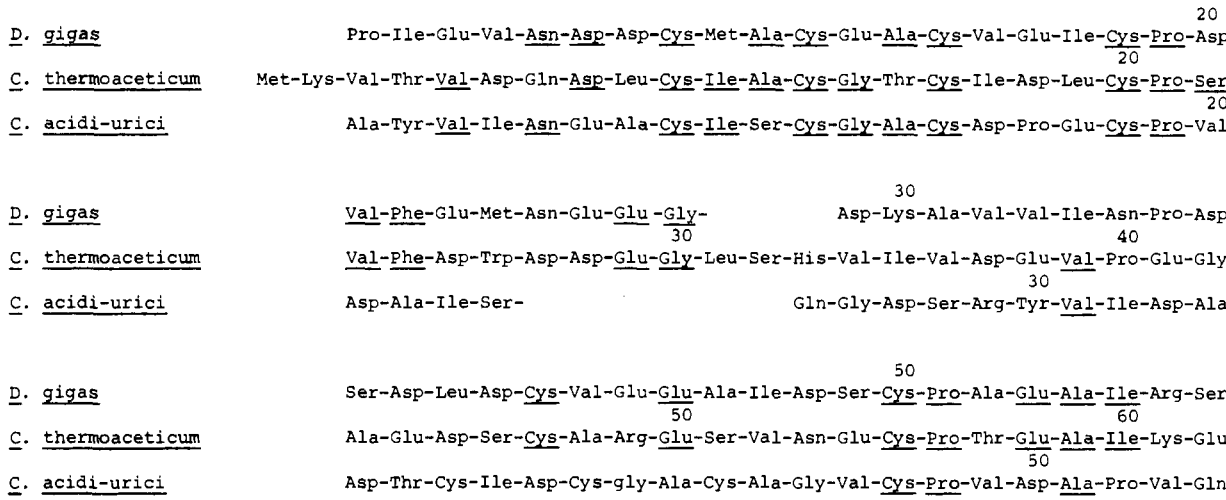

FIGURE 2: Comparisons of the amino acid sequences of the ferredoxins from *C. thermoaceticum*, *C. acidi-urici* (Rall et al., 1969), and *D. gigas* (Bruschi, 1979). Due to the different total amino acid contents, alignments were made with the Cys-Pro sequences located in the carboxy- and amino-terminal regions of the proteins.

residues 3–49), while peaks 2 and 3 were evidently shortened versions of this peptide. All three fragments exhibited the amino-terminal sequence of Val-Thr-Val-Asp-Gln (residues 3–7 of the complete sequence) and were not sequenced further. Peak 4 (T-4) was a 12-residue fragment containing lysine (residues 50–61) and was sequenced once by using solid-phase techniques and twice by using the spinning-cup procedure (see Figure 1 and Table I). Peak 5 contained two dipeptides: Met,Lys and Glx,Val. The Met,Lys peptide corresponded to the first two residues of the native protein, while the Glx,Val peptide proved to be the carboxy-terminal dipeptide based on carboxypeptidase Y digestion followed by hydrazinolysis (see Materials and Methods). Carboxypeptidase Y released valine quantitatively from the ^{14}C-labeled S-carboxymethylated, whole protein, but no further degradation took place at pH 5.5 or 7.0, either with or without 6 M urea. Hydrazinolysis of the carboxypeptidase Y treated protein showed the penultimate residue was a glutamic acid. Yields were 90–95% for carboxypeptidase Y release of valine and 55–60% for glutamic acid, following hydrazinolysis.

Discussion

The amino acid sequence of the 4Fe–4S ferredoxin from *C. thermoaceticum* is presented in Figure 1 and agrees with the amino acid composition for the purified protein. This composition differed slightly from that originally reported by Yang et al. (1977) in that three prolines were identified rather than four and four serines, rather than three.

During the course of this sequencing work, it became obvious that this ferredoxin was resistant to enzymatic and chemical cleavages. Tryptic, chymotryptic, and *Staphylococcus aureus* protease digestions, under what would be considered standard conditions, were not successful. Tryptic digests were finally performed at substrate to enzyme ratios of 9:1 (w/w), but clean fragmentation and yields (25–35%) were poor. Chemical cleavages were also largely unsuccessful. Iodosobenzoic acid in 4 M guanidine hydrochloride (Mahoney & Hermodson, 1979) and cyanogen bromide in heptafluorobutyric and formic acids (Ozols & Gerard, 1977) did not give measurable cleavage at the tryptophan residue (Trp-26). This ferredoxin is very insoluble at pH values less than 3–4 and does not dissolve in 25–100% acetic acid. Limited acid hydrolysis in 0.03 N HCl, 110 °C, in vacuo, from 48 h and 12 N HCl, 37 °C, for 48 h did not produce significant cleavages, as the protein formed a clear, gellike precipitate. The observed stability to degradation correlated well with its high thermal stability (stable for 60 min at 80 °C; Yang et al., 1977) and may be related to the very acidic nature of this ferredoxin. Of the 18 acidic residues by amino acid analysis (9 glutamates and 9 aspartates), only 2 amide residues were found in the sequence work (Asn-53 and Gln-7).

The ferredoxin from *C. thermoaceticum* exhibits a number of unusual properties. Clostridial ferredoxins studied to date have 54–56 amino acid residues and two [4Fe–4S] clusters. The ferredoxin from *C. thermoaceticum* has 63 residues and only a single [4Fe–4S] cluster (Yang et al., 1977). Furthermore, it contains some uncommon residues for clostridial and clostridial-like ferredoxins. Tryptophan (Trp-26) has not been previously reported for any clostridial or related ferredoxins. Histidine (His-33), for clostridial ferredoxins, is found only in the thermophilic species, *C. tartarivorum* and *C. thermosaccharolyticum* (Devanathan et al., 1969); however, histidine is also detected in the nonthermophilic, clostridial-like ferredoxin from *Peptostreptococcus elsdenii* (Yasunobu & Tanaka, 1973). Methionine (Met-1) is not reported for the clostridial ferredoxins but is observed in *P. elsdenii* (Yasunobu & Tanaka, 1973), also at residue 1. Arginine (Arg-49) has only been previously measured in the clostridial ferredoxin from *C. acidi-urici* (Lovenberg et al., 1963). Additionally, leucine (Leu-9, -19, and -31) is found as a single residue in ferredoxins from *C. cylindrosporum* and *C. tetanomorphum* (Lovenberg et al., 1963) but is not present in other clostridial ferredoxins or those from *Peptococcus aerogenes* (Tsunoda et al., 1968) and *P. elsdenii* (Yasunobu & Tanaka, 1973).

The primary structure of the ferredoxin from *C. thermoaceticum* is also strikingly different from other clostridial and related ferredoxins. Comparison of these sequences is presented in Figure 2 and Table II. The high degree of homology between typical 8Fe–8S ferredoxins is shown in Table II by using *C. tartarivorum* ferredoxin as the standard. The *C. thermoaceticum* ferredoxin shows a low degree of homology when compared to the other clostridial and clostridial-like ferredoxins. A somewhat higher level of homology is actually observed for the comparisons to the 4Fe–4S ferredoxins from *D. gigas* and the aerobe, *B. stearothermophilus*. Figure 2 shows that the similarities which do exist are predominantly associated with the sulfhydryl groupings in the amino-terminal and carboxy-terminal regions. In addition, the sequence of the *C. thermoaceticum* ferredoxin shows little similarity between the amino- and carboxy-terminal halves of the protein,

Table II: Homology in the Primary Sequence of Ferredoxins from Various Bacterial Sources

	C. thermoaceticum			*C. tartarivorum*			
	NH_2[a]	COOH[a]	total	NH_2[a]	COOH[a]	total	total residues
C. acidi-urici	8	4	12	16	16	32	55
C. butyricum	8	3	11	16	17	33	55
C. pasteurianum	7	3	10	16	17	33	55
C. tartarivorum	8	5	13				55
C. thermosaccharolyticum	8	5	13	25	28	53	55
P. aerogenes	10	3	13	16	15	31	54
P. elsdenii	8	5	13	20	16	36	54
D. gigas	11	7	18	8	3	11	57
B. stearothermophilus	13	6	19	8	6	14	81
C. thermoaceticum				8	5	13	63

[a] Indicates the number of identical residues in corresponding positions in the amino- and carboxy-terminal halves when the sequences are aligned as in Figure 2. Ferredoxins from the first seven species listed are of the 8Fe–8S type and the remainder are 4Fe–4S.

as is observed for the typical clostridial ferredoxin (Yasunobu & Tanaka, 1973).

Bacillus stearothermophilus contains a ferredoxin having a single [4Fe–4S] cluster and just four cysteine residues (Hase et al., 1976). Although this protein has 81 residues, the sequence shows the cysteines located in positions similar to those found for the 8Fe–8S ferredoxins: Cys-11, -14, and -17 at the amino-terminal region and Cys-61 in the Cys-Pro sequence in the carboxy-terminal region. This indicates that the [4Fe–4S] cluster of *B. stearothermophilus* is held in a similar manner to the amino-terminal cluster (cluster 1) of *P. aerogenes* (Adman et al., 1973). *C. thermoaceticum* and *D. gigas* ferredoxins have single [4Fe–4S] clusters and six sulfhydryls, and both have the same Cys-Pro sequence for their terminal sulfhydryl (residues 50–51 for *D. gigas* and 55–56 for *C. thermoaceticum*). It appears that, as proposed by Hase et al. (1976), this Cys-Pro sequence is critical to a functional, low-potential [4Fe–4S] cluster. All anaerobic and aerobic ferredoxins containing such a cluster(s) show this Cys-Pro sequence. *P. aerogenes*, based on the three-dimensional data of Adman et al. (1973), has Cys-Pro sequences located at residues 19–20 and 45–46 which serve to bind the iron–sulfur cluster located at the opposite end of the protein. Thus, this sequence serves to bend the amino acid chain away from the iron–sulfur center. Both *D. gigas* and *C. thermoaceticum* have a second Cys-Pro sequence in the amino-terminal region even though they lack two clusters.

Thermal stability has been proposed to be related to a number of different factors. One, based on ferredoxin sequences, correlates thermal stability and an increased number of internal glutamic acid residues in the sequence (Hase et al., 1976). *C. thermoaceticum* ferredoxin has seven such glutamates (excluding the penultimate glutamate) and is consistent with the established pattern. Perutz & Raidt (1975) compared two clostridial ferredoxins, which differ sequentially by just two amino acid residues, by computer-generated, three-dimensional structures based on the sequence data. They proposed that a salt bridge involving His-2 and Gln-44 of the ferredoxin from *C. thermosaccarolyticum* could largely account for the additional thermal stability of this protein when compared to the ferredoxin from *C. tartarivorum*. The latter contains a glutamic acid rather than a glutamine at residue 44 and does not form a salt bridge with His-2. Histidine is reported at residue 2 in thermophilic ferredoxins from *C. tartarivorum* (Tanaka et al., 1971), *C. thermosaccharolyticum* (Yasunobu & Tanaka, 1973), and the aerobe, *Thermus thermophilus* (Sato et al., 1981). Lysine is present at the identical position in the ferredoxins from *B. stearothermophilus* (Hase et al., 1976) and, as presented here, *C. thermoaceticum*. All represent thermophilic ferredoxins with a positively charged group located at residue 2. This, in itself, is not sufficient to impart thermostability as shown by the thermolabile ferredoxin from *P. elsdenii* which has a histidine at position 2 (Yasunobu & Tanaka, 1973). It becomes important to ascertain the placement of the positively charged residue in the ferredoxin's three-dimensional structure to clearly determine its relationship to thermostability.

It is important to note that a second ferredoxin has been isolated from *C. thermoaceticum* (Elliott & Ljungdahl, 1982) which has properties consistent with those of a typical clostridial ferredoxin: 57 residues and two low-potential [4Fe–4S] clusters. Thus, *C. thermoaceticum* has two, distinct ferredoxins which presumably have different cellular roles.

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

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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.

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

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