

A-I milano apoprotein. Isolation and characterization of a cysteine-containing variant of the A-I apoprotein from human high density lipoproteins.

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Research Article

A recently discovered familial lipoprotein disorder is characterized by reduced plasma levels of high density lipoproteins (HDL) and elevated triglyceride levels. The clinical aspects of this disorder are presented in an accompanying article (Franceschini et al. 1980. *J. Clin. Invest.* 66: 892-900). The apoprotein content of the HDL isolated from these patients differed markedly from that of normal HDL in that three apoprotein bands not previously described in man were present as major protein components. As determined by sodium dodecyl sulfate (SDS) gel electrophoresis, the relative molecular weights (Mr) of these new apoprotein bands were 55,000, 35,000, and 28,000. Although the Mr 28,000 apoprotein coelectrophoresed with authentic A-I on SDS polyacrylamide gels and showed immunochemical identity with the A-I apoprotein when tested with monospecific apo-A-I antiserum, it contained two amino acid residues, cysteine and isoleucine, which were not present in the amino acid sequence of normal human apo-A-I. This variant form of the A-I apoprotein was designated the A-IMilano apoprotein and denoted A-Icys. By virtue of the presence of cysteine (2 mol/mol A-Icys), the A-Icys apoprotein was capable of forming intermolecular disulfide bonds, and dimer formation of A-Icys produced the Mr 55,000 apoprotein. The Mr 35,000 apoprotein was composed of two different subunits, A-Icys and A-II. By analogy to the apo(E--A-II) complex, which also occurs in human HDL, this mixed [...]

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A-I_{Milano} Apoprotein

ISOLATION AND CHARACTERIZATION OF A CYSTEINE-CONTAINING VARIANT OF THE A-I APOPROTEIN FROM HUMAN HIGH DENSITY LIPOPROTEINS

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ABSTRACT A recently discovered familial lipoprotein disorder is characterized by reduced plasma levels of high density lipoproteins (HDL) and elevated triglyceride levels. The clinical aspects of this disorder are presented in an accompanying article (Franceschini et al. 1980. *J. Clin. Invest.* 66: 892–900). The apoprotein content of the HDL isolated from these patients differed markedly from that of normal HDL in that three apoprotein bands not previously described in man were present as major protein components. As determined by sodium dodecyl sulfate (SDS) gel electrophoresis, the relative molecular weights (M_r) of these new apoprotein bands were 55,000, 35,000, and 28,000. Although the M_r 28,000 apoprotein coelectrophoresed with authentic A-I on SDS polyacrylamide gels and showed immunochemical identity with the A-I apoprotein when tested with monospecific apo-A-I antiserum, it contained two amino acid residues, cysteine and isoleucine, which were not present in the amino acid sequence of normal human apo-A-I. This variant form of the A-I apoprotein was designated the A-I_{Milano} apoprotein and denoted A-I_{cys}. By virtue of the presence of cysteine (2 mol/mol A-I_{cys}), the A-I_{cys} apoprotein was capable of forming intermolecular disulfide bonds, and dimer formation of A-I_{cys} produced the M_r 55,000 apoprotein. The M_r 35,000 apoprotein was composed of two different subunits, A-I_{cys} and A-II. By analogy to the apo(E—A-II) complex, which also occurs in human HDL, this mixed disulfide complex was designated as the apo(A-I_{cys}—A-II) complex. The A-I_{Milano} (A-I_{cys})

is the first example of a variation in the primary sequence of a protein of plasma lipoproteins.

INTRODUCTION

Normal human plasma high density lipoproteins (HDL, $d = 1.063$ – 1.21)¹ contain, as their major protein components, the A-I (M_r 28,000), dimeric A-II (M_r 17,000), and C ($M_r < 10,000$) apoproteins. Together, the A-I and A-II apoproteins usually account for >85% of the total HDL protein, with the concentration of apo-A-I being two to four times that of apo-A-II. Low concentrations of apo-D, apo-E (M_r 37,000), and the apo(E—A-II) complex (M_r 46,000) also occur in human HDL (1–5). By contrast, HDL isolated from the plasma of nephrotic or diabetic patients, or from patients treated with amphotericin B have been shown to contain the normal HDL apoproteins and also to be enriched in two other apoproteins, the threonine-poor apoproteins (6).

Recently, a male patient (D.V.) has come to our attention (7) whose plasma HDL and apo-A-I levels are markedly reduced compared with normal values, but who did not exhibit any of the characteristic clinical features of the known HDL deficiency disorders: Tangier disease (8–13), lecithin:cholesterol acyltransferase (LCAT) deficiency (14), and “fish-eye” disease (15). The familial HDL deficiency disorder of the patient (D.V.) and affected children is characterized by hypertriglyceridemia and normocholesterolemia (7). The HDL reduction is associated with an HDL-cholesterol, which is 20–30% of the value obtained for normal subjects, and a marked reduction in plasma A-I apoprotein

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¹ Abbreviations used in this paper: DTNB, 5,5'-dithiobis (2-nitrobenzoic acid); HDL, high density lipoprotein; LCAT, lecithin:cholesterol acyltransferase.

levels (>50% reduced in D.V.). Clinically, there is no evidence of the deposition of cholesterol in tissues and no evidence of splenomegaly, corneal opacification, neuropathy, uremia, or atherosclerotic vascular disease. Furthermore, there are no gross abnormalities of LCAT activity (7). It appears that a unique biochemical feature of this new familial lipoprotein disorder is the abnormal HDL apoprotein content, which differed significantly from that of normal HDL in that three apoproteins that have not been previously described in man are present as major apoprotein components. The isolation and characterization of these variant HDL apoproteins are presented in this report.

METHODS

Preparation of lipoproteins. Plasma containing 1 mg/ml EDTA was obtained from the fasted subject. The plasma lipoproteins were separated into density classes by sequential ultracentrifugation in a type 60 titanium rotor (Beckman Instruments, Inc., Fullerton, Calif.). The $d < 1.063$ fraction was prepared by raising the density of plasma to $d = 1.063$ with potassium bromide and centrifuging for 16 h at 59,000 rpm. The 1.063–1.21 density fraction was isolated by centrifugation at 59,000 rpm for 36 h followed by a recentrifugation at $d = 1.21$ for 24 h. In some cases, the blood for lipoprotein isolation was treated with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) by withdrawing blood directly into the DTNB reagent (50 mg/ml DTNB, 0.1 M phosphate buffer, pH 7.0) in a ratio of 40 parts blood to 5 parts DTNB reagent. Immediately after preparation of the plasma, an additional amount of DTNB was added (0.2 ml reagent/10 ml plasma). Lipoprotein density fractions were dialyzed against 0.01% EDTA, pH 7.4. Protein was determined by the method of Lowry et al. (16), using bovine albumin as a reference.

Heparin-Sepharose affinity column chromatography of the 1.063–1.21 density fraction was performed as described (5). Subfractionation was carried out on 4.9 mg of lipoprotein protein at 4°C. The bound fractions, HDL-with E and the β -subclass, were eluted with 0.095 and 0.29 M NaCl, respectively (method A) (5). The separated subclasses were dialyzed against 0.01% EDTA, pH 7.0.

Isolation and characterization of the apoproteins. HDL apoproteins were prepared by CHCl_3 :MeOH (2:1, vol/vol) delipidation of the lyophilized HDL fraction. Apoproteins for sodium dodecyl sulfate (SDS) gel electrophoresis were incubated at 37°C for 2 h in a 2.5 mM Tris-buffer containing glycine (pH 8.2) and 0.3% SDS. Reduction of disulfide bonds was accomplished by addition of β -mercaptoethanol (Sigma Chemical Co., St. Louis, Mo.) to the incubation mixture to give a final concentration of 125 mM. SDS polyacrylamide gel electrophoresis was performed on 11% gels using the Tris/glycine buffer system of Stephens (17). Apoprotein molecular weights were determined by SDS gel electrophoresis using human serum albumin, leucine aminopeptidase, human apo-E, human apo-A-I, and reduced and nonreduced human apo-A-II as reference proteins. The albumin was reduced with β -mercaptoethanol before electrophoresis. Normal apo-A-I was isolated from delipidated control HDL by gel filtration on a 3-m Sephadex G-200 column (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) equilibrated with 4 M guanidine and 0.2 M Tris (pH 8.0).

The patient's (D.V.) HDL apoproteins were isolated by preparative SDS gel electrophoresis as described by Stephens (17). The apo(A-I_{cys}—A-II) subunits were isolated by prepara-

tive SDS gel electrophoresis after the complex was reduced and alkylated. To 1 mg of apo(A-I_{cys}—A-II) in 1 ml of 0.2 M phosphate buffer (pH 8.0) was added 30 μ l of β -mercaptoethanol, and the mixture was incubated for 4 h at room temperature. Alkylation of the reduced subunits was performed in the dark for 30 min at 4°C by the addition of 100 mg of iodoacetamide. Excess reagents were removed by dialysis (5 mM NH_4HCO_3), and the sample was lyophilized.

Isoelectric focusing was performed on 6-cm, 5% polyacrylamide gels containing 8 M urea and 2% Ampholine, pH 3.5–7.0 (LKB Instruments, Bromma, Sweden), according to the method of Pagnan et al. (18) with the buffer system modified as previously described (4). Immunodiffusion was performed according to the method of Ouchterlony as described by Clausen (19).

Amino acid analysis of the purified apoproteins was performed on a Beckman 121M automatic amino acid analyzer (Beckman Instruments, Inc.) after acid hydrolysis of the protein in 6 N HCl for 22 h at 110°C under a nitrogen atmosphere. Cysteic acid was determined after performic acid oxidation of the apoproteins, using the method of Moore (20). Glycine is a constituent of the buffer in the preparative polyacrylamide gel electrophoresis procedure used to isolate the variant apoproteins. We have observed that trace amounts of glycine may not be removed from the gel-isolated apoproteins even after extensive dialysis and may be detected during amino acid analyses; therefore, the content of glycine may be inaccurate.

RESULTS

Examination of the SDS gel pattern of the HDL fraction ($d = 1.063$ –1.21) from the plasma of the patient (D.V.) revealed a much more complex pattern than that observed with the HDL from normal subjects (Fig. 1). This abnormal pattern was characterized by the presence of four prominent protein components, which were designated as bands I, II, III, and A-II. As determined by SDS gel electrophoresis, using proteins of known molecular weights as standards, the apparent molecular weights of bands I, II, and III were 55,000, 35,000, and 28,000, respectively. Unlike normal HDL, in which only the disulfide-linked A-II dimer was affected by mercaptoethanol treatment, reduction of the abnormal HDL with mercaptoethanol had a dramatic effect on the SDS gel pattern. As shown in Fig. 1, the M_r 55,000 (I) and M_r 35,000 (II) apoproteins were no longer present after disulfide reduction. Associated with their loss was an increase in the staining intensity of the M_r 28,000 apoprotein (band III). As expected, the A-II was also reduced to its lower molecular weight subunits ($M_r \sim 8,500$) after reduction with mercaptoethanol.

To characterize bands I, II, and III, we isolated them in pure form by preparative SDS gel electrophoresis and determined the effect of disulfide reduction on each one (Fig. 2). Reduction of the band I apoprotein converted this M_r 55,000 protein to a single M_r 28,000 band, suggesting that band I consisted of two M_r 28,000 subunits linked by one or more disulfide linkages. After reduction, band II (M_r 35,000) was converted into

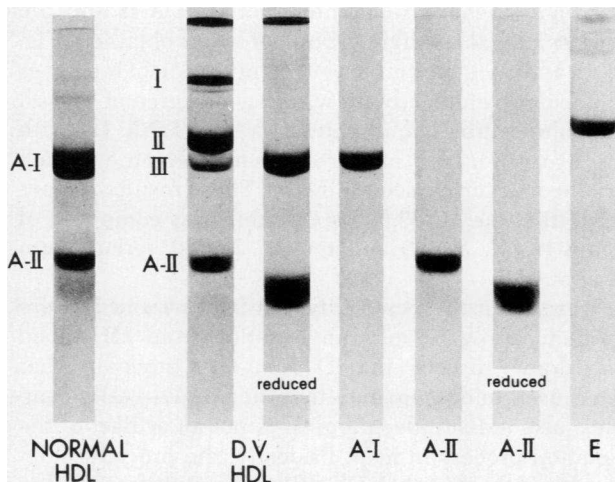


FIGURE 1 SDS-polyacrylamide gel electrophoresis of HDL from a normal subject and patient D.V. Bands I, II, and III correspond to the M_r 55,000, 35,000, and 28,000 apoproteins, respectively. Purified apo-A-I, A-II, and E are shown for comparison. Reduced samples were treated with 1% mercaptoethanol.

two subunits, one with a molecular weight of 28,000 and the other with a molecular weight of <10,000 (Fig. 2), demonstrating that band II was composed of two different subunits joined by one or more disulfide linkages. Reduction of the band III apoprotein did not affect its migration on SDS gels.

The presence of cysteine intermolecular linkages in the I and II apoproteins, suggested by mercaptoethanol reduction, was confirmed by amino acid analysis (Table I). In addition, the band III apoprotein was also shown to contain cysteine. Based on a molecular weight

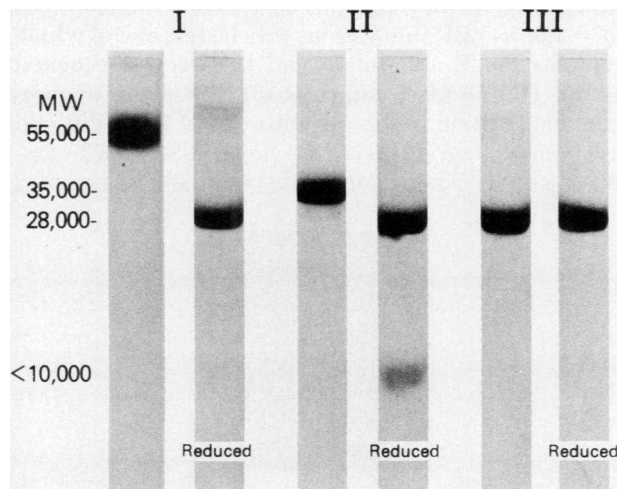


FIGURE 2 SDS-polyacrylamide gel electrophoresis of the purified band I, II, and III apoproteins, isolated from D.V. HDL, showing the effect of mercaptoethanol reduction on each.

of 28,000, band III contained 2 mol cysteine/mol protein. The amino acid compositions of bands I and III were very similar, supporting the suggestion that band I was composed of two band III subunits joined by one or more disulfide bonds. Interestingly, the amino acid composition of these apoproteins closely resembled that of human apo-A-I except for the presence of cysteine and isoleucine (Table I). There was also a slight increase in the glycine content (see Methods). Furthermore, immunochemical studies clearly demonstrated cross-reactivity of the M_r 55,000, 35,000, and 28,000 bands with monospecific antiserum to the normal human apo-A-I obtained from HDL. When tested against the apo-A-I antiserum by the technique of immunodiffusion, the band I, II, and III apoproteins showed not only a strong precipitant reaction, but also complete precipitin lines of identity with the authentic A-I standard (Fig. 3). These results indicate a strong immunochemical relationship between bands I, II, and III and the A-I apoprotein, despite the known absence of cysteine and isoleucine residues in normal human apo-A-I sequence.

The relationships between the abnormal HDL apoproteins (I, II, and III) and the A-I apoprotein were characterized further by isoelectric focusing on polyacrylamide gels. As shown in Fig. 4, the apo-A-I region of the normal HDL gel indicated the presence of two major A-I components. The A-II apoprotein focused

TABLE I
Amino Acid Composition of Abnormal HDL Apoproteins and Normal A-I Apoprotein

	Apoprotein			
	I	II	III	Normal A-I
	mol %			
Asp	9.1	8.9	9.1	9.6
Thr	4.3	4.9	4.2	4.1
Ser	7.0	6.9	7.2	6.3
Glu	19.1	20.4	19.3	20.2
Pro	4.6	4.5	4.4	4.3
Gly	6.6	5.1	6.3	4.5
Ala	8.0	7.7	8.0	8.3
½Cys	0.8*	0.7*	1.0*	0*
Val	4.9	5.0	4.6	4.7
Met	0.2	0.8	0.2	0.6
Ileu	0.6	0.2	0.6	0
Leu	14.2	14.1	14.4	15.6
Tyr	2.7	3.2	2.7	2.7
Phe	2.6	2.7	2.3	2.3
Lys	7.9	8.9	7.8	8.4
His	1.7	1.3	1.7	1.9
Arg	5.7	4.7	5.9	6.6

Results are the average of duplicate determinations on two preparations.

* Determined after performic acid oxidation.

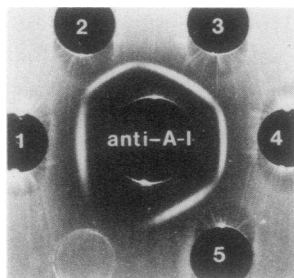


FIGURE 3 Immunodiffusion demonstrating immunochemical identity of the band I, II, and III apoproteins with the A-I apoprotein on reaction with monospecific antiserum to the A-I apoprotein (anti-A-I). Positions 1 and 5, apo-A-I; position 2, band I; position 3, band II; position 4, band III.

as a single band with a pI of ~ 4.2 . The abnormal HDL apoproteins showed, in addition to the A-II band, a series of bands with pIs slightly more acidic than those of A-I. After reduction, the abnormal HDL apoprotein focusing pattern remained different from that of normal HDL, with the major components in the A-I region focusing at slightly more acidic pIs than control A-I (Fig. 4). After reduction with mercaptoethanol, the isolated I and II apoproteins focused into identical patterns in the A-I region of the gel. The patterns were identical to that of band III, which was not affected by mercaptoethanol treatment. These results confirmed that band III was a subunit of bands I and II.

In addition, the reduction of band II resulted in the appearance of a band with a pI identical to that of the A-II apoprotein (see arrow, Fig. 4), suggesting that the A-II apoprotein was the lower molecular weight subunit of the M_r 35,000 protein (band II). To confirm the identity of the band II subunits, we reduced and alkylated band II and isolated the two subunits by preparative SDS gel electrophoresis. When tested against monospecific apo-A-II antiserum using the technique of immunodiffusion, a precipitin reaction demonstrat-

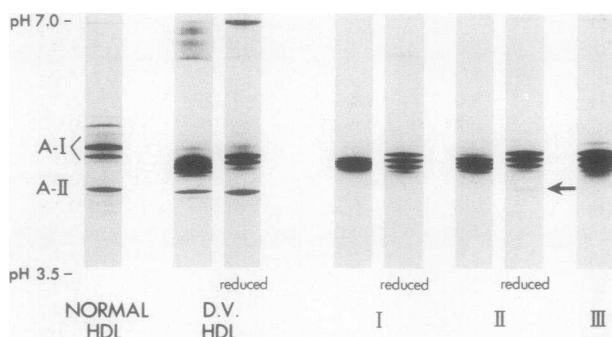


FIGURE 4 Isoelectric focusing on 5% polyacrylamide gels containing 8 M urea and 2% Ampholine, pH 3.5–7.0, of the apoproteins from normal and D.V. HDL, and of the purified band I, II, and III apoproteins from D.V. HDL. Reduced samples were treated with 1% mercaptoethanol.

ing immunochemical identity between A-II and the lower molecular weight subunit of II was obtained (Fig. 5). In addition, the amino acid composition of the lower molecular weight subunit was in good agreement with the composition of authentic apo-A-II (Table II), with the exception of a higher content of glycine, which may be inaccurate (see Methods). These results demonstrate that the M_r 35,000 apoprotein was composed of apo-A-II (M_r 8,500) and the M_r 28,000 variant form of apo-A-I.

To rule out the possibility that bands I and II were formed during lipoprotein isolation from III, blood was drawn directly into DTNB. This procedure has been used to demonstrate that the apo(E—A-II) complex, also a disulfide complex, is not an artifact of the isolation process in man. Basically, the procedure depends on the fact that DTNB reacts with free sulfhydryl groups and would prevent disulfide linkages from forming in vitro. The concentration of DTNB present in the whole blood was approximately threefold greater than necessary to oxidize all of the available free sulfhydryl groups, as determined by Ellman (21). After preparation of serum from the DTNB-treated blood, additional DTNB was added so that a 40-fold excess was present during the lipoprotein isolation. As determined by SDS-gel electrophoresis (data not shown), the HDL isolated from the DTNB-treated blood contained both the I and II apoproteins. There was, however, a reduction in the total amount of the band I and II apoproteins, suggesting that at least some of the bands I and II complexes might form during isolation. Examination of the $d < 1.063$ lipoproteins did not reveal significant concentrations of the three abnormal apoproteins (I, II, and III) in this density fraction.

Recently we have reported the use of heparin-Sepharose affinity chromatography to separate human HDL ($d = 1.063$ –1.21) into various subclasses, one of which contains the E apoprotein and has been designated as the HDL-with E subclass (5). Since the apoprotein composition of the patient's (D.V.) HDL differed from normal, we compared the heparin-Sepharose pro-

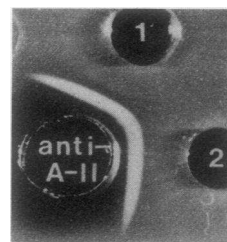


FIGURE 5 Immunodiffusion showing identity between the lower molecular weight subunit of the band II apoprotein and the A-II apoprotein on reaction with prepared monospecific antiserum to the A-II apoprotein (anti-A-II). Position 1, band II subunit; position 2, apo-A-II.

TABLE II
Amino Acid Composition of the Lower Molecular Weight
Subunit of Band II and the A-II Apoprotein

	M _r 8,500 subunit*	Apo-A-II
	mol %	
Asp	4.9	4.9
Thr	7.3	7.4
Ser	8.5	7.9
Glu	20.5	21.7
Pro	5.0	5.5
Gly	7.5	4.6
Ala	6.7	7.1
½Cys	ND†	0.6
Val	6.7	7.1
Met	0.5	0.6
Ileu	1.7	1.2
Leu	10.0	10.4
Tyr	4.3	4.9
Phe	4.6	4.6
Lys	11.0	10.9
His	0.4	0.2
Arg	0.4	0.4

* Duplicate determinations on a single preparation.

† ND, not determined.

file of the D.V. HDL with the profile from normal HDL to determine whether the D.V. HDL contained the E apoprotein. Subfractionation of the D.V. HDL resulted in a profile (Fig. 6) that was quantitatively similar to that obtained with normal HDL, with the HDL-without E, HDL-with E, and the β -subclasses accounting for 84.5, 10.3, and 5.2% of the total HDL protein, respectively. These values differ slightly from those obtained from a population of six normal individuals (5): HDL-without E, 91.8 ± 2.2 ; HDL-with E, 6.3 ± 1.6 ; and β , $2.0 \pm 1.2\%$ (\pm SD). Apoprotein analysis by SDS gel electrophoresis of the various subclasses (Fig. 6) showed that the HDL-without E was composed of the bands I, II, and III apoproteins, as well as the apo-A-II. In addition to these apoproteins, the HDL-with E contained the E apoprotein, which was partially masked by the band II apoprotein on the gel of a non-reduced sample, but was readily apparent after mercaptoethanol reduction (Fig. 6). The β -subclass was composed primarily of the B apoprotein, with trace amounts of the I, II, III, and E apoproteins.

DISCUSSION

Examination of the HDL fraction ($d = 1.063$ – 1.21) of the plasma from a patient with low total plasma HDL levels and hypertriglyceridemia (subject D.V., described in the accompanying article [7]), revealed the presence of three apoproteins that have not been previously described in man. As determined by SDS gel

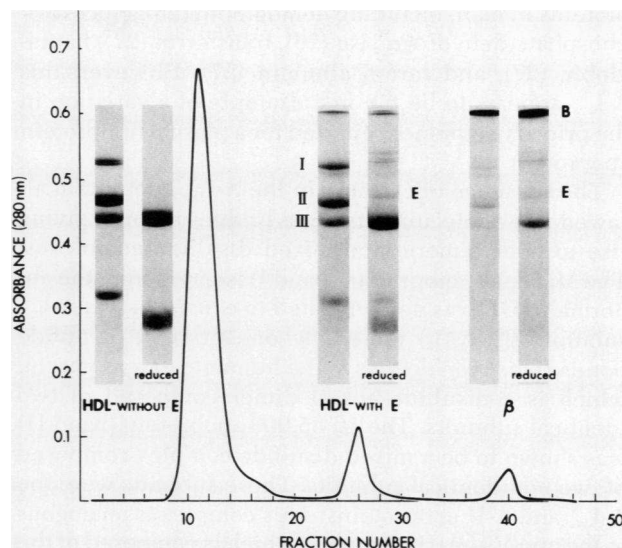


FIGURE 6 Heparin-Sepharose affinity chromatography of D.V. HDL and SDS-polyacrylamide gel electrophoresis of the HDL-without E, HDL-with E, and the β -subclasses. Approximately 5 mg of HDL protein in 0.005 M Tris-Cl, pH 7.4, containing 0.05 M NaCl and 0.025 M $MnCl_2$, was applied to the column (1.0×30 cm). The column was operated at a rate of 24 ml/h and 3.6-ml fractions were collected. The unbound HDL-without E subclass (fractions 8–18) was eluted in the column void volume with the Tris-NaCl- Mn^{+2} -containing buffer. The column was then eluted in a stepwise manner. As fraction 12 was being collected, the eluting buffer was changed to a buffer containing 0.095 M NaCl and no $MnCl_2$. This resulted in the elution of the HDL-with E subclass (fractions 24–30). An increase in the NaCl concentration to 0.29 M NaCl at fraction 27 eluted the β -subclass (fractions 38–42).

electrophoresis, the apparent molecular weights of these apoproteins, designated as bands I, II, and III, were 55,000, 35,000, and 28,000, respectively. Based on both its mobility on SDS gels and its immunochemical reactivity with A-I antiserum, band III initially appeared to be identical to the normal human A-I apoprotein; however, the amino acid composition of band III differed from that of normal apo-A-I in that band III contained cysteine (2 mol/mol protein) and isoleucine (~ 1 mol/mol protein), both of which are absent from the primary amino acid sequence of the normal human A-I apoprotein (22–24). Apart from differences in cysteine and isoleucine, the amino acid compositions of band III and A-I were quite similar. The fact that A-I and band III revealed immunochemical identity indicated that a significant amount of sequence homology existed between these apoproteins. Thus, band III represented a variant form of the A-I apoprotein with apparent amino acid substitutions of cysteine and isoleucine. Therefore, we have designated band III as the A-I_{cys}(A-I_{Milano}) apoprotein. Several examples of amino acid substitutions are known to occur with other

proteins in man, including hemoglobin (25), glucose 6-phosphate dehydrogenase (26), transferrin (27), haptoglobin (27), and serum albumin (27). However, this A-I_{cys} appears to be the first example of a variation in the primary sequence reported for a plasma lipoprotein apoprotein.

The presence of cysteine in the A-I_{cys} apoprotein allowed intermolecular disulfide bridges to form, giving rise to both dimeric and mixed disulfide complexes. The M_r 55,000 apoprotein (band I) isolated from the abnormal HDL was demonstrated to consist of two A-I_{cys} subunits ([A-I_{cys}]₂) joined by one or more disulfide bonds. This is analogous to the human A-II apoprotein, which is a disulfide-linked dimer composed of two identical subunits. The M_r 35,000 apoprotein (band II) was shown to be a mixed disulfide complex composed of two nonidentical subunits. These subunits were the A-I_{cys} and A-II apoproteins. This complex is analogous to the apo(E—A-II) complex, which is composed of the E and A-II apoproteins (4). Therefore, we propose to designate band II as the apo(A-I_{cys}—A-II) complex.

As previously shown with normal human HDL, the D.V. HDL could be subfractionated by heparin-Sepharose affinity chromatography into subclasses, which corresponded to normal HDL subclasses (5). The D.V. HDL included the HDL-with apo-E and HDL-without apo-E subclasses.

The mechanism responsible for the occurrence of A-I_{cys} apoprotein in this subject remains an open question. It is possible that the A-I_{cys} may be present in very low concentration in normal plasma as a result of A-I gene duplication, but is not normally detected because of the predominance of the A-I apoprotein. Its detection in the D.V. HDL may then be the consequence of an altered A-I metabolism (accelerated catabolism), which leads to an absence of normal A-I in HDL. Alternatively, this subject may have a mutation in the coding process for a normal A-I molecule. The familial nature of the defect has been established (7).

The dimeric and mixed disulfide forms of the A-I_{cys} apoprotein are easily detected on SDS gels because they migrate in positions different from those of other known apoproteins, and they are affected by mercaptoethanol reduction. Thus, for large-scale screening studies, these characteristics are suitable markers to indicate the presence of the A-I_{cys} apoprotein. Such studies are currently underway to determine both the occurrence and distribution of the variant A-I in the whole kindred of the test subject, as well as in other populations.

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