Evidence that Lp[a] contains one molecule of apo[a] and one molecule of apoB: evaluation of amino acid analysis data

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Abstract Amino acid analysis was performed on four Lp[a] preparations to evaluate whether or not the amino acid data was consistent with Lp[a] containing one molecule of apolipoprotein[a] [apo(a)] linked to one molecule of apoB-100. Amino acid analysis was carried out in duplicate on a Beckman model 121 amino acid analyzer. Apo[a] size was determined by a high-resolution agarose gel electrophoretic method that provides an estimate of apo[a] kringle 4 repeats. When Lp[a] was assumed to contain one apo[a] and one apoB molecule per particle, the average absolute bias between the expected molar percentage of each amino acid, as based on the known sequence of apo[a] and apoB, and the obtained molar percentage ranged from 2 to 3.5%. In contrast, by assuming two molecules of apo[a] and one of apoB per Lp[a] particle, the bias between the expected and observed molar percentage ranged from 8.5% to 10%, and by assuming one apo[a] and two apoB the bias ranged from 8.8% to 11.4%. Comparison of Lp[a] concentrations, calculated from six stable amino acids and the Lp[a] composition predicted from the known sequence, was in excellent agreement (bias ranging from 0.3% to 0.9%) with the Lp[a] concentration calculated from the sum of the amino acid concentrations, when Lp[a] was assumed to contain one molecule of apo[a] and one molecule of apoB. However, there was poor agreement (7.4% to 8.4% bias) when it was assumed that Lp[a] contains two molecules of apo[a] and one molecule of apoB. These results indicate that the evaluated Lp[a] preparations contain one apo[a] per Lp[a] particle. Evaluation of amino acid analysis data provides a relatively simple approach to determine the molar ratio of apoB to apo[a] in Lp[a] and provides evidence that Lp[a] contains one molecule of apo[a] and one molecule of apoB.–**Albers, J. J., H. Kennedy, and S. M. Marcovina.** Evidence that Lp[a] contains one molecule of apo[a] and one molecule of apoB: evaluation of amino acid analysis data. *J. Lipid Res.* 1996. **37:** 192–196.

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It is commonly believed that the protein molecule of Lp[a] consists of one molecule of apoB-100 linked by a sulfhydryl bond to one molecule of apo[a] of variable size (1). Apo[a] is formed by three distinct structural domains, each with a high degree of homology with the corresponding domain of plasminogen. Apo[a] is formed by an inactive protease domain, one copy of a kringle 5 domain, and variable copies of the kringle 4 domain (2). Apo[a] kringle 4 (K4) can be subdivided into 10 distinct kringle types based on differences in amino acid sequence (3). Each kringle has six cysteine residues that form three intramolecular disulfide bonds. Only one kringle, the K4 type 9, has an extra cysteine that is not involved in the intra-kringle bond. Available evidence strongly suggests that the extra cysteine of kringle 4 type 9 (Cys^{67}) forms a disulfide bridge with Cys^{3734} of apoB-100 (1, 4). In addition to the apoB–apo[a] disulfide bond, multiple strong non-covalent interactions including multiple van der Walls contacts and interactions between lysine-binding kringles of apo[a] and lysine residues in apoB, have been proposed to hold the Lp[a] molecule together (1, 4, 5). In contrast to the prevailing view that only one apo[a] is linked to apoB, results of two physicochemical studies have suggested that Lp[a] particles contain two molecules of apo[a] linked to one molecule of apoB-100 (6, 7). Recently, we have provided immunochemical evidence that the ratio of apoB to apo[a] is constant in Lp[a] (8). This report evaluates the use of Lp[a] amino acid analysis data to evaluate the molar ratio of apo[a] to apoB in Lp[a].

MATERIALS AND METHODS

Lp[a] was isolated from fresh plasma of fasting donors by sequential density ultracentrifugation followed by gel filtration (8). Each donor was preselected to contain a

Abbreviations: apo, apolipoprotein; Lp[a], lipoprotein[a]; apoB, apolipoprotein B-100; PIR, protein information resource.

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single apo[a] isoform. Plasma was immediately separated by low-speed centrifugation at 4°C, and the preservatives NaN_3 (0.2 g), phenylmethysulfonyl fluoride (174.2 mg), ε-aminocaproic acid (26.5 g), and aprotinin (10,000 KIU) were added to each liter of plasma containing 0.1 g of Na_2 EDTA. Gel immunodiffusion studies, using rabbit anti-human apoE and rabbit anti-human apoA-I, indicated that less than 5% of the total protein of these Lp[a] preparations was either apoE or apoA-I. As an internal standard, 75 nmol of norleucine was added to 250 μl of purified Lp[a]. Duplicate samples of intact Lp[a], without lipid extraction, were hydrolyzed in 6 N HCl, 0.05% mercaptoethanol, 0.02% phenol, for 20 h at 115°C in sealed evacuated hydrolysis tubes. Analyses were carried out on a Beckman model 7300 amino acid analyzer equipped for single column methodology using Beckman's sodium buffer system and Beckman's System Gold software for data analysis. To compensate for destruction by acid hydrolysis, serine values were increased by 10% and threonine by 5%. All amino acid analysis values were corrected for possible sample transfer losses or possible inaccurate volume measurements during sample application, by calculations taking into account recovery values for the norleucine internal standard. Expected amino acid composition was derived from apo[a] and apoB sequence data obtained from the Protein Information Resource (PIR) database. The differences between the sequence-derived and the amino acid analysis-derived Lp[a] concentration was tested with the *t*-test for dependent samples.

The apo[a] size isoforms were determined by a high-resolution agarose gel electrophoretic method (9). The apo[a] isoform numerical identification was converted to number of kringle 4 contained in each isoform by the previously established relationship between number of kringle 4 repeats determined by pulse-field gel electrophoresis and migration of apo[a] isoforms in agarose gel (8).

RESULTS

The amino acid composition of Lp[a] derived from the amino acid analysis was compared to the combined expected amino acid composition of apo[a] and apoB-100 obtained from the amino acid sequence of both proteins. The average absolute bias between the expected molar percentage for each amino acid and that observed by amino acid analysis ranged from 2.0 to 3.5% if Lp[a] was assumed to contain one molecule of apo[a] and one of apoB-100 (**Table 1**). In contrast, the average absolute bias between the expected and observed amino

TABLE 1. Amino acid composition of lipoprotein[a] assuming one apo[a] and one apoB-100 in Lp[a]

	Separate Lp[a] Preparations with 20 Kringle 4									Lp[a] Preparation with 18 Kringle 4		
Amino	1			2			3			4		
Acid	Seq.	AAA	% bias	Seq.	AAA	% bias	Seq.	AAA	% bias	Seq.	AAA	% bias
A	6.30	6.25	-0.8	6.30	6.90	9.5	6.30	6.45	2.4	6.22	6.68	7.4
R	4.25	4.37	2.8	4.25	4.28	0.7	4.25	4.23	-0.5	4.19	4.16	-0.7
D[a]	9.44	9.46	0.2	9.44	9.50	0.6	9.44	9.50	0.6	9.52	9.53	0.1
C	2.30	2.31	0.4	2.30	2.30	0.0	2.30	2.32	0.9	2.20	2.21	0.5
E[b]	11.42	11.73	2.7	11.42	11.86	3.9	11.42	11.68	2.3	11.42	12.02	5.3
G	5.39	5.46	1.3	5.39	5.61	4.1	5.39	5.48	1.7	5.33	5.71	7.1
H	2.61	2.60	-0.4	2.61	2.61	0.0	2.61	2.71	3.8	2.61	2.49	-4.6
I	4.59	4.23	-7.8	4.59	4.14	-9.8	4.59	4.27	-7.0	4.71	4.19	-11.0
L	8.66	8.85	2.2	8.66	8.75	1.0	8.66	8.80	1.6	8.86	8.78	-0.9
K	5.35	5.42	1.3	5.35	5.00	-6.5	5.35	5.35	0.0	5.52	5.31	-3.8
M	1.75	1.76	0.6	1.75	1.75	0.0	1.75	1.77	1.1	1.75	1.76	0.6
F	3.40	3.31	-2.6	3.40	3.10	-8.8	3.40	3.35	-1.5	3.51	3.32	-5.4
P	6.09	6.12	0.5	6.09	6.09	0.0	6.09	6.15	1.0	5.91	5.94	0.5
S	8.46	8.25	-2.5	8.46	8.21	-3.0	8.46	8.22	-2.8	8.48	8.33	-1.8
T	8.29	8.25	-0.5	8.29	8.23	-0.7	8.29	8.27	-0.2	8.19	8.20	0.1
W	1.38	1.38	0.0	1.38	1.37	-0.7	1.38	1.39	0.7	1.36	1.37	0.7
Y	4.59	4.61	0.4	4.59	4.56	-0.7	4.59	4.41	-3.9	4.48	4.35	-2.9
V	5.74	5.60	-2.4	5.74	5.73	-0.2	5.74	5.65	-1.6	5.73	5.63	-1.7
Average absolute bias[c]			2.0			3.5			2.1			3.8

Amino acid values expressed in mole %. Seq. is the amino acid composition computed from the combined amino acid sequence of apo[a] and apoB-100 obtained from the Protein Information Resource database. AAA is the amino acid composition obtained from amino acid analysis except for C, M, P, and W which was computed from the known sequence of apo[a] and apoB-100. Percent bias equals [(AAA mole % - Seq. mole %)/Seq. mole %] × 100.

[a]D represents the sum of D and N.

[b]E represents the sum of E and Q.

[c]The computation of average absolute bias excludes the value for C, M, P, and W as these values were computed from the sequence data.

TABLE 2. Amino acid composition of lipoprotein[a] assuming two apo[a] and one apoB-100 in Lp[a]

Amino Acid	Separate Lp[a] Preparations with 20 Kringle 4									Lp[a] Preparation with 18 Kringle 4		
	1			2			3			4		
	Seq.	AAA	% bias	Seq.	AAA	% bias	Seq.	AAA	% bias	Seq.	AAA	% bias
A	6.48	6.01	7.8	6.48	6.64	-2.4	6.48	6.20	4.5	6.37	6.44	-1.1
R	4.71	4.20	12.1	4.71	4.12	14.3	4.71	4.09	15.2	4.64	4.01	15.7
D[a]	8.93	9.09	-1.8	8.93	9.14	-2.3	8.93	9.12	-2.1	9.03	9.18	-1.6
C	3.12	3.40	-8.2	3.12	3.37	-7.4	3.12	3.43	-9.0	3.01	3.26	-7.7
E[b]	11.33	11.28	0.4	11.33	11.42	-0.8	11.33	11.19	1.3	11.33	11.58	-2.2
G	5.78	5.25	10.1	5.78	5.40	7.0	5.78	5.27	9.7	5.72	5.50	4.0
H	2.64	2.50	5.6	2.64	2.51	5.2	2.64	2.59	1.9	2.65	2.40	10.4
I	3.80	4.07	-6.6	3.80	3.99	-4.8	3.80	4.09	-7.1	3.94	4.04	-2.5
L	7.29	8.50	-14.2	7.29	8.42	-13.4	7.29	8.47	-13.9	7.51	8.46	-11.2
K	4.16	5.21	-20.2	4.16	4.81	-13.5	4.16	5.13	-18.9	4.36	5.11	-14.7
M	1.77	1.93	-8.3	1.77	1.91	-7.3	1.77	1.95	-9.2	1.77	1.91	-7.3
F	2.68	3.18	-15.7	2.68	2.99	-10.4	2.68	3.22	-16.8	2.81	3.20	-12.2
P	7.21	7.88	-8.5	7.21	7.79	-7.4	7.21	7.93	-9.1	7.01	7.58	-7.5
S	8.38	7.93	5.7	8.38	7.90	6.1	8.38	7.92	5.8	8.40	8.02	4.7
T	9.09	7.93	14.6	9.09	7.92	14.8	9.09	7.94	14.5	8.97	7.90	13.5
W	1.64	1.79	-8.4	1.64	1.77	-7.3	1.64	1.80	-8.9	1.63	1.76	-7.4
Y	5.18	4.43	16.9	5.18	4.39	18.0	5.18	4.27	21.3	5.04	4.19	20.3
V	5.82	5.38	8.2	5.82	5.52	5.4	5.82	5.42	7.4	5.81	5.43	7.0
Average absolute bias[c]				10.0			8.5			10.0		8.7

Amino acid values expressed in mole %. Seq. is the amino acid composition computed from the combined amino acid sequence of apo[a] and apoB-100 obtained from the Protein Information Resource database. AAA is the amino acid composition obtained from amino acid analysis except for C, M, P, and W which was computed from the known sequence apo[a] and apoB-100. Percent bias equals [(AAA mole % - Seq. mole %)/Seq. mole %] × 100.

[a]D represents the sum of D and N.

[b]E represents the sum of E and Q.

[c]The computation of average absolute bias excludes the value for C, M, P, and W since these values were computed from the sequence data.

acid compositions was significantly higher, ranging from 8.5 to 10.0%, when Lp[a] was assumed to contain two molecules of apo[a] and one of apoB-100 per particle (**Table 2**). The absolute biases between the assumed one to one apo[a]-apoB ratio and the assumed two to one apo[a]-apoB-100 ratio were significantly different ($P < 0.001$). The average absolute bias between the expected and observed amino acid compositions was also significantly higher, ranging from 14.9 to 17.6%, when Lp[a] was assumed to contain three apo[a] and one apoB-100 per particle and it ranged from 8.8% to 11.2%, when Lp[a] was assumed to contain one apo[a] and two apoB-100 per particle (data not shown). The superior agreement between the expected and observed amino acid composition when it was assumed that Lp[a] contains a single molecule of apo[a] and apoB per particle, strongly suggests that the Lp[a] preparations examined were formed by one molecule of apo[a] and one of apoB-100.

Due to the lack of full recovery of some of the amino acids during acid hydrolysis, we next compared the relative percentages of the six stable and fully recovered amino acids (A, D, E, L, K, F), as calculated from the sequence data, to the relative percentages obtained from the amino acid analysis. The average percentage difference and the sum of the absolute percentage differences is presented in **Table 3.** The absolute % differences between the expected and observed percentages are significantly different assuming one apo[a] per particle versus two apo[a] per particle ($P < 0.001$).

We then calculated and compared the Lp[a] concentrations on the basis of these six stable amino acids and the combined amino acid composition of apo[a] and apoB-100 obtained from the reported sequence, to the Lp[a] concentrations computed from the summation of the amino acid concentrations obtained from the amino acid analysis (**Table 4**). The agreement between the two different approaches for computation of Lp[a] concen-

TABLE 3. Differences between amino acid analysis and sequence data for six amino acids

Assumption	Sample	Kringle 4 No.	Average Absolute % Difference	Sum Absolute % Difference
1 Apo[a] per apoB	1	20	0.2	1.4
	2	20	0.6	3.6
	3	20	0.2	1.0
	4	18	0.6	3.5
Mean			0.4	2.4
2 Apo[a] per apoB	1	20	1.5	9.0
	2	20	1.0	5.8
	3	20	1.4	8.6
	4	18	0.9	5.5
Mean			1.2	7.2

The average and sum of the absolute % difference was calculated from percentages derived for six amino acids (A, D, E, L, K and F).

TABLE 4. Lp[a] concentrations obtained from amino acid analysis

Assumption	Sample	Kringle 4 No.	Lp[a] Concentration		% Bias
			Sequence	AAA	
			nmol/L		
1 Apo[a] per apoB	1	20	355.3	356.5	0.3
	2	20	550.4	551.7	0.2
	3	20	596.5	601.9	0.9
	4	18	461.6	465.1	0.8
2 Apo[a] per apoB	1	20	272.0	295.4	7.9
	2	20	420.9	454.4	7.4
	3	20	456.6	498.3	8.4
	4	18	358.0	387.1	7.5

Lp[a] concentration was calculated from the sequence on the basis of the concentration of A, D, E, L, K and F and the combined amino acid composition of apo[a] and apoB-100 obtained from the Protein Information Resource database. The AAA Lp[a] concentration was obtained from the summation of the amino acid concentration from the amino analysis except for C, M, P, and W which were computed from the known sequence of apo[a] and apoB-100.

trations was excellent (0.3 to 0.9% bias) when Lp[a] was assumed to contain a single molecule of apo[a] and apoB-100 (Table 4). The differences between the predicted and observed Lp[a] concentrations were not statistically significant (P = 0.065). In contrast, when Lp[a] was assumed to contain two apo[a] and one apoB-100, the Lp[a] concentrations from the two computational approaches differed significantly (P = 0.003). Differences ranged from 7.4 to 8.4% bias (Table 4). These results indicate that these four Lp[a] preparations contained one apo[a] for each apoB-100. Additionally, when Lp[a] was assumed to contain two apo[a] per particle, the Lp[a] concentration was computed to be about 17% lower than that obtained assuming one apo[a] per particle (Table 4).

Because the apo[a] amino acid composition will vary with the number of apo[a] K4 repeats, an error in the estimate of kringle 4 number would contribute to an error in Lp[a] concentration. Additionally, variation in the number of sialic acid residues may potentially alter the migration of apo[a] in the agarose gel. The effect of K4 number on the computation of Lp[a] concentration obtained from amino acid analysis was consequently evaluated (**Table 5**). A one kringle difference in the number of kringles of each of the Lp[a] preparations would contribute to only a 0.9 to 1% difference in Lp[a] concentration. Therefore, a slight error in the estimate of the number of kringles does not contribute to a significant error in the estimate of Lp[a] concentration.

DISCUSSION

The evaluation of amino acid analysis data provides a relatively simple approach to determine the molar ratio of apoB to apo[a] in Lp[a] preparations. However, several potential limitations of this approach need to be discussed. To simplify the calculations, we limited our evaluations to donors who exhibited a single apo[a] band even though less than 20% of the general population has a single apo[a] isoform. In order to apply our approach to individuals with two apo[a] isoforms, it would be necessary to determine the molar ratio of the two apo[a] polymorphs. Scanning densitometry of the immunoblots would permit an estimate of the relative proportions of the isoforms and therefore could make it feasible to extend our approach to Lp[a] preparations containing two isoforms. Another potential limitation relates to the likely possibility that a proportion of the isolated Lp[a] particle contains apolipoproteins other than apoB and apo[a]. Blanco-Vaca et al. (10) reported the presence of apoE and apoA-I in Lp[a] preparations and Bard et al. (11) also found the presence of apoC. We and others have reported that apo[a] is associated with triglyceride-rich lipoprotein particles particularly in non-fasting subjects fed a fat-rich meal (12, 13). However, all our donors were fasting and were not hypertriglyceridemic. Additionally, Lp[a] preparations used in this study were isolated using molecular sieve chromatography in the presence of 0.2 mol/L proline to minimize nonspecific protein adsorption (8). Although Lp[a] has been reported to interact non-covalently with LDL (14), it is highly unlikely that our preparations contain bound LDL because proline, which is known to disrupt LDL-Lp[a] interactions (14), was used in the isolation procedure. The excellent agreement between the expected and observed amino acid composition suggests that other proteins were not a major constituent of these Lp[a] preparations. Lp[a] particles containing a significant proportion of one or more apolipoproteins other than apo[a] or apoB does not preclude the

TABLE 5. Effect of kringle number on computation of Lp[a] concentration obtained from amino analysis

Sample	Kringle 4 No. 19[a]	Kringle 4 No. 20[b]	Kringle 4 No. 21[a]
	Lp[a] Concentration		
	nmol/L		
1	359.8	356.5	353.3
2	557.1	551.7	546.5
3	607.5	601.9	596.4
	Kringle 4 No. 17[a]	Kringle 4 No. 18[b]	Kringle 4 No. 19[a]
4	469.7	465.1	460.6

The Lp[a] concentration was calculated on the basis of the concentration of A, D, E, L, K and F and the combined amino acid composition of apo[a] and apoB-100 obtained from the Protein Information Resource database.

[a]Assumed Kringle 4 number.

[b]Kringle 4 number based on mobility of apo[a] in agarose gel electrophoresis.

data analysis approach taken here. The contribution of other apolipoproteins to the Lp[a] amino acid composition could be considered in the analysis. If Lp[a] was assumed to contain either one molecule of apoE or apoA-I, in addition to apo[a] and apoB-100, the agreement between the expected and observed amino acid composition would not change significantly because the presence of one of these small apolipoproteins represents only 4% of the protein mass of Lp[a].

Fless et al. (7) have recently reported that the protein moiety of each of four Lp[a] preparations evaluated in their study consisted of a complex of two molecules of apo[a] and one molecule of apoB. These conclusions were made from results of three different physicochemical methods. The reason for the different findings of these authors could relate to fundamental structural differences in the Lp[a] preparations but more likely may relate to the different approaches to assess the molar ratio of apo[a] to apoB in Lp[a]. In the study of Fless et al. (7), the protein content of apoB and apo[a] was determined by the method of Lowry et al. (15). Based on the comparison between SDS-Lowry and amino acid analysis of Lp[a] preparations, we have found that the Lowry procedure overestimates the Lp[a] protein concentration. Because the extinction coefficient of Lp[a] protein reflects the combination of the absorption of apoB and apo[a], based on our results the Lowry method also significantly overestimates the apo[a] protein mass. Contrary to our finding, Fless et al. (7) reported that the apo[a] protein was highly underestimated by the Lowry procedure and therefore used the factor 1.4 to correct the Lowry value of each apo[a] preparation to the absolute protein concentration. Thus, an error in the estimate of protein mass may be one of the contributing factors that account for the discrepancy between the finding of the present study and that previously reported (7).

In conclusion, our results provide strong chemical evidence for the presence of a single apo[a] polypeptide for each Lp[a] particle.

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