Structural Comparison of Human Apolipoproteins B-48 and B-100†

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ABSTRACT: In this study we have investigated the structural relationship between human apolipoproteins B-48 and B-100 by comparing protein structure and by comparing nucleotide sequence from intestinal and hepatic cDNA clones. Sequences from intestinal and hepatic cDNA were identical over the entire distance analyzed (7194 bases), which is more than required to code for B-48. The amino-terminal amino acid sequences from intact B-48 and B-100 proteins were also identical over the entire distance analyzed (16 residues). Additional protein homology was evaluated by the combined techniques of peptide mapping and immunoblotting. Purified B-48 and B-100 were each digested with three different endoproteases, and the resulting peptides were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Peptide bands were then detected by silver stain and by Western blotting with antisera against specific regions of B-48 and B-100. The resulting patterns suggest that B-48 is extensively homologous with the amino-terminal portion of B-100. We have identified only four peptides from B-48 (at least one in each digest) that are absent from the parallel digests of B-100. These peptides appear to arise from the ultimate carboxyl terminus of B-48 and appear to be totally homologous with a region located near the center of B-100. Our observations suggest that mature, circulating B-48 is homologous over its entire length (estimated to be between 2130 and 2144 amino acid residues) with the amino-terminal portion of B-100 and contains no sequence from the carboxyl end of B-100. This corresponds to about 47% of the B-100 amino acid sequence or about 240 000 daltons of protein.

Very low density lipoproteins (VLDL), low-density lipoproteins (LDL), and chylomicrons, from human plasma, each contain a very high molecular weight protein component traditionally termed apolipoprotein B (apoB).[1] Previously, we have shown that the apoB found on chylomicrons differs from that found on VLDL and LDL with respect to apparent molecular weight and amino acid composition (Kane et al., 1980). To describe these proteins, we employed a centile system of nomenclature that is based on relative apparent molecular weights estimated by SDS–PAGE. In this system, the molecular weight of each form of apoB is expressed as a percentage of the molecular weight of the largest form. The largest, and normally most abundant form, termed B-100 (apparent M_r 549 000), is found on the lipoproteins of hepatic origin, the VLDL and LDL. The other form, termed B-48 (apparent M_r 264 000), is found on the lipoproteins of intestinal origin, the chylomicrons. This pattern is observed in most animal species, although a protein that appears to be B-48 is secreted by the liver in rodents (Elovson et al., 1981; van't Hooft et al., 1981).

In humans, as in most species, B-48 and B-100 are considered to be metabolically distinct; they are synthesized independently in separate organs and generally experience separate metabolic fates. Nonetheless, they also appear to be functionally and structurally related. For example, both proteins are essential for the synthesis and secretion of their respective particles. In addition, both proteins appear to possess a very high affinity for lipid, which may be responsible for their inability to transfer among particles. Together, these properties suggest that both B-48 and B-100 may play roles in the formation or maintenance of particle structure. Moreover, B-48 and B-100 have a number of antigenic determinants in common, suggesting that they share a degree of sequence homology (Marcel et al., 1982; Curtiss & Edgington, 1982), and it has been proposed, on the basis of an immunochemical study of a genetic polymorphism in human B-48 and B-100, that both proteins are coded by the same structural gene (Young et al., 1986), suggesting that B-48 could arise by proteolytic cleavage of B-100 or by differential splicing of the B-100 transcript.

Recently, the complete nucleotide sequence of hepatic cDNA coding for B-100 has been reported (Chen et al., 1986; Knott et al., 1986). These studies have shown that mature, circulating B-100 is composed of a single polypeptide chain 4536 amino acid residues in length. Immunochemical evidence suggests that most of the homology between B-48 and B-100 could be limited to a specific region of B-100 (Marcel et al., 1982). Using an antiserum against a synthetic peptide, we have shown that at least part of this homology is associated

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[1] Abbreviations: apoB, apolipoprotein B; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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with the amino-terminal region of B-100 (Protter et al., 1986a). However, additional immunochemical evidence has been presented suggesting that B-48 also contains sequence from the carboxyl end of B-100 (Hospattankar et al., 1986). The presence, in B-48, of sequence from these two widely separated regions of B-100 would imply that the structural relationship between B-48 and B-100 is not a simple one and that B-48 probably arises by differential splicing of mRNA.

At this time, however, the precise structure of B-48 protein has not been determined so that the structural relationship between B-48 and B-100 is not known. Elucidation of this relationship is essential for understanding the mechanism responsible for the formation of B-48 and for understanding the structural basis for metabolic differences between B-48 and B-100. Therefore, in this study, we have conducted a detailed structural comparison of B-48 and B-100 using the techniques of amino acid sequence analysis, one-dimensional peptide mapping, and immunoblotting. In addition, we have compared nucleotide sequence from intestinal and hepatic cDNA coding for apoB and have related these findings to the protein data.

Materials and Methods

Isolation of Liver and Intestine ApoB Clones. Human clones for apolipoprotein B were isolated from intestine and liver λ gt 10 cDNA libraries and characterized essentially as described previously (Protter et al., 1986a,b). Briefly, in order to obtain apoB cDNA clones that map 3′ to the intestine and liver clones that were previously characterized, the libraries were screened with unique synthetic oligonucleotides, whose sequences map to the 3′-end of the previously determined cDNA sequence. The oligonucleotide probes were made radioactive by treatment with T4 polynucleotide kinase and [γ-^{32}P]ATP and used to screen the cDNA libraries as described (Protter et al., 1986b). The cDNA clones were subcloned into m13 Mp18 or Mp19, and their nucleotide sequence was determined by the dideoxy method (Sanger et al., 1977) with the universal primer (P-L Biochemicals) or unique 17-base oligonucleotides to prime the reaction.

Isolation of Lipoproteins. Lipoproteins of $\rho < 1.006$ g/cm^3 were isolated from the serum of individuals with mixed lipemia and from the lymph of individuals undergoing lymphophoresis. Low-density lipoproteins were isolated from the serum of normal donors. Microbial and chemical degradation of lipoproteins was prevented by the addition of gentamycin sulfate (0.1 mg/mL), sodium azide (0.05%), and EDTA (0.05%, pH 7.0) to the original samples and to all ultracentrifugation media. The $\rho < 1.006$ g/cm^3 lipoproteins were isolated by ultracentrifugation of serum or lymph at 30 000 rpm in a 40.3 rotor at 12 °C for 18 h. The supernatant fraction was recentrifuged under the same conditions. Low-density lipoproteins were isoalted in the density range 1.024–1.063 g/cm^3. Solvent densities were adjusted with solid anhydrous potassium bromide. Ultracentrifugation at each step was performed at 38 000 rpm in a 40.3 rotor at 12 °C for 18 h. Centrifugation at the higher density was performed twice, and the purified LDL were dialyzed against 0.15 M sodium chloride. All isolated lipoproteins were brought to a protein concentration of 2 mg/mL with 0.15 M sodium chloride and were stored at −20 °C.

Purification of Apolipoproteins. Apolipoproteins B-48 and B-100 were isolated essentially as described previously (Hardman & Kane, 1986). Briefly, a sample of $\rho < 1.006$ g/cm^3 lipoprotein (2 mg/mL) was delipidated in 20 volumes of ethanol–diethyl ether (3:1) for 15 min at 22 °C. The precipitated protein was dispersed in 4% SDS, 0.1 M sodium phosphate, pH 7.2, and 1% 3-mercapto-1,2-propanediol to achieve a final protein concentration of 2 mg/mL. The protein was allowed to dissolve at 22 °C and was then heated to 100 °C for 1 min. The slightly turbid solution was clarified by ultracentrifugation at 38 000 rpm in a 40.3 rotor at 22 °C for 45 min.

The clear protein solution was applied to a 2.2 × 80 cm Sepharose CL-6B column eluted with 0.1% SDS, 0.025 M sodium phosphate, pH 7.2, 0.05% sodium azide, and 0.05% EDTA at 22 °C. Fractions containing B-48 and B-100 were pooled and concentrated to 3 mL by ultrafiltration with an Amicon PM-30 membrane. The concentrated sample was rechromatographed under the same conditions. Fractions containing mostly B-48 were pooled and concentrated to 1.5 mL as above. Likewise, fractions containing mostly B-100 were pooled and concentrated.

The partially purified samples of B-48 and B-100 were each combined with glycerol and 3-mercapto-1,2-propanediol to achieve final concentrations of 10% and 1%, respectively, and were heated to 100 °C for 15 s. Each sample was then subjected to preparative SDS–PAGE with a 2.3% resolving gel (Hardman & Kane, 1980). Fractions containing pure B-48 and B-100 were each pooled and were stored at −20 °C. Apolipoproteins B-26 and B-74, for immunization, were isolated from LDL in a similar fashion.

Sequence Analysis. Purified B-48 and B-100 were each concentrated by ultrafiltration with an Amicon PM-30 membrane. Each sample was then transferred to 0.1% SDS–0.1 M ammonium bicarbonate by repeated ultrafiltration. Amino-terminal amino acid sequence analysis was performed on an Applied Biosystems Model 470A protein microsequencer (Hunkapiller & Hood, 1983).

Digestion with Endoproteases. Purifed B-48 and B-100 were each concentrated by ultrafiltration to 1 mg/mL. Each sample was then applied to a 1 × 60 cm Sepharose CL-6B column eluted with 0.05% SDS–0.15 M Tris-HCl, pH 8.4, to transfer to the new buffer.

Parallel digests of B-48 and B-100 were performed as follows with three different endoproteinases (Boehringer-Mannheim). For digests with endoproteinase Glu-C from *Staphylococcus aureus* V8 (SP), 48 μg of B-48 and 100 μg of B-100 were each combined with 2.6 μg of enzyme in a total volume of 740 μL of the column buffer. Digestion was carried out at 37 °C for 160 min. For digests with elastase from porcine pancreas (EL), 48 μg of B-48 and 100 μg of B-100 were each combined with 6.8 μg of enzyme in a total volume of 940 μL of column buffer. Digestion was carried out at 22 °C for 20 min. For digests with endoproteinase Lys-C from *Lysobacter enzymogenes* (LC), 48 μg of B-48 was combined with 2.8 units of enzyme, and 100 μg of B-100 was combined with 4 units of enzyme, each in a total volume of 740 μL of column buffer. Digestion was carried out at 37 °C for 80 min. At the end of each digestion, the samples were heated to 100 °C for 15 s and were stored at −20 °C.

Analytical SDS–PAGE. Peptides from the enzyme digests were separated by SDS–PAGE with a 4.5–20% linear gradient slab gel and a discontinuous buffer system (Laemmli, 1970). Slabs measuring 14 × 14 cm and incorporating 10 lanes per gel were used throughout. Slabs intended for silver staining were cast 0.75 mm thick and were loaded with 1.86 μg of B-48 digest or 3.86 μg of B-100 digest per lane. Slabs intended for Western blotting were cast 1.5 mm thick and were loaded with 3.72 μg of B-48 digest or 7.72 μg of B-100 digest per lane. Prior to electrophoresis, samples were combined with glycerol, sodium mercaptoacetate, and bromophenol blue to final con-

centrations of 10%, 1%, and 0.005%, respectively.

Immunoblotting. For dot blots, known quantities of protein were applied to nitrocellulose filters, unoccupied binding sites were blocked with gelatin, and the filters were incubated with various antisera. For detection, a horseradish peroxidase–goat antirabbit conjugate (Bio-Rad) was used.

For Western blots, intact proteins or peptides were transferred to nitrocellulose filters following SDS–PAGE, as described previously (Towbin et al., 1979). Unoccupied binding sites were blocked with 5% nonfat dry milk at 42 °C. The filters were incubated with various antisera, washed, incubated with ^{125}I-labeled staphylococcal protein A at 0.1 μCi/mL (Amersham), washed again, and exposed to Kodak X-Omat film. Incubation and wash steps were performed at 22 °C, and exposure to the film was carried out at −70 °C.

Preparation of Antsera against Synthetic Peptides. Synthetic peptides were prepared by solid-phase peptide synthesis on the Applied Biosystems peptide synthesizer 430A, as described (Protter et al., 1986a). The crude peptides were purified by ion exchange and HPLC on C18 columns. The peptide homogeneity was judged to be greater than 98% by HPLC analysis, and the amino acid composition was consistent with the peptide structure. The peptides were conjugated to keyhole limpet hemocyanin and then injected into rabbits as described (Potter et al., 1986a).

B-100 Affinity Chromatography. A sample of intact LDL containing 10 mg of protein in 6.5 mL of 0.5 M sodium chloride–0.1 M sodium bicarbonate was combined with 0.5 g of CNBr–Sepharose (Pharmacia) that had been washed previously in 1 mM HCl. This mixture was incubated at 22 °C for 24 h. The resin was then packed in a column 0.6 cm in diameter and was washed extensively with 0.2 M Tris-HCl, pH 8.0. The column was then washed with 5% SDS–0.1 M sodium phosphate, pH 7.2, to denature the LDL and was equilibrated with 0.05% Tween-20, 0.5 M sodium chloride, and 0.02 M Tris-HCl, pH 7.5. Fifty microliters of antiserum against B-48 was applied to the column and was eluted with equilibration buffer at a flow rate of 1.3 mL/h. Fractions were collected at 5-min intervals and were assayed for the presence of rabbit IgG on dot blots with a horseradish peroxidase–goat antirabbit conjugate (Bio-Rad). Fractions were also assayed for their ability to recognize intact B-48 and B-100 on dot blots and to recognize peptides on Western blots.

Purification of Peptides for Sequence Analysis. Three samples of B-48, each containing 600 μg of protein, were digested with endoproteinases SP, EL and LC as described above. One sample of B-100, containing 1 mg of protein, was digested with EL. After digestion, each sample was concentrated to a volume of 1.5 mL by ultrafiltration with an Amicon PM-10 membrane. Glycerol and 3-mercapto-1,2-propanediol were added to achieve final concentrations of 10% and 1%, respectively, and the peptides were separated by preparative SDS–PAGE (Hardman & Kane, 1980). Peptides from the SP digest of B-48 and from the EL digest of B-100 were each separated on a 5% resolving gel. Peptides from the EL and LC digests of B-48 were separated on 6.5% and 7.5% resolving gels, respectively. After isolation, the purity of each peptide was tested on analytical SDS gels stained with silver. In preparation for amino-terminal amino acid sequence analysis, purified peptides were each concentrated and transferred to 0.01% SDS–0.1 M ammonium bicarbonate by repeated ultrafiltration with a PM-10 membrane.

Results and Discussion

Previously, we have presented the nucleotide sequence of human cDNA clones coding for the amino-terminal 184 000 daltons of apoB. In this study, we have extended the characterization of cDNA clones from both liver and intestine in order to compare the structures of mRNA coding for apoB from these two tissues and to aid in the characterization of B-48 protein. Figure 1 shows the nucleotide sequence and derived amino acid sequence of liver and intestine cDNA clones encoding amino acid residues 1644–2370 of apoB. This sequence was represented in both hepatic and intestinal clones (data not shown). Except for minor nucleotide differences, which could be attributed to sequencing or cloning artifacts, the liver and intestine forms appear to be identical. Recently, the complete nucleotide and derived amino acid sequence of B-100, as determined through the analysis of cDNA clones of hepatic origin, has been presented (Chen et al., 1986; Knott et al., 1986). The intestine-derived sequence presented here is essentially identical. Thus, at the RNA level, no differences could be seen between apoB produced from the two tissues.

Table I: Amino-Terminal Amino Acid Sequences Obtained from Apolipoproteins B-100, B-48, and B-26[a]

B-100	GluGluGluMetLeuGluAsnValSerLeuVal---ProLysAspAla
B-48	GluGluGluMetLeuGluAsnValSerLeuVal---ProLysAspAlaThr
B-26	GluGluGluMetLeuGluAsnValSerLeuVal---ProLysAspAlaThrLys
from cDNA	GluGluGluMetLeuGluAsnValSerLeuValCysProLysAspAlaThrLys

[a] Also shown is the amino acid sequence predicted from a cDNA clone coding for the amino terminus of B-26.

At the protein level, homology between B-48 and B-100 was initially studied by amino-terminal amino acid sequence analysis of the intact proteins. Both proteins, isolated by preparative SDS–PAGE, were tested for purity on analytical SDS gels stained with silver. Under the conditions described, B-48 and B-100 each appeared as a single band with no other bands visible (data not shown). The sequences from intact B-48 and B-100 (Table I) were identical over the distance analyzed (16 residues), establishing that these proteins are entirely homologous at their amino termini. These sequences appear to represent the native amino-terminal sequences of the mature, circulating forms of B-48 and B-100, on the basis of our previous observation, in hepatic cDNA, that the corresponding nucleotide sequence is located immediately downstream from typical signal peptide and initiation sequences (Protter et al., 1986a). It follows from these findings that the primary structures of B-48 and B-100 are aligned as shown in Figure 2. The remainder of this paper describes experiments to determine how far this homology extends beyond the amino-terminal region.

Next, B-48 and B-100 were compared by means of one-dimensional peptide mapping. Purified B-48 and B-100 were partially digested with three different proteolytic enzymes: endoproteinase Glu-C from *Staphylococcus aureus* V8 (SP), elastase from porcine pancrease (EL), and endoproteinase Lys-C from *Lysobacter enzymogenes* (LC). Peptides from the digests were subjected to analytical SDS–PAGE, and the resulting patterns were compared after silver staining and Western blotting. In each case, the concentrations of B-48 and B-100 were matched on a molar basis. Conditions of digestion were developed empirically to obtain peptides ranging in size from 10^3 to 10^5 daltons.

Typical patterns revealed by silver stain are shown in Figure 3. From Figure 3 it is apparent that digests of B-100 contain many peptides that are absent from the parallel digests of B-48, confirming that B-100 contains regions that are not homologous with B-48. In addition, a number of other bands appear to be present in digests of both B-48 and B-100, consistent with previous reports of homology between the two proteins.

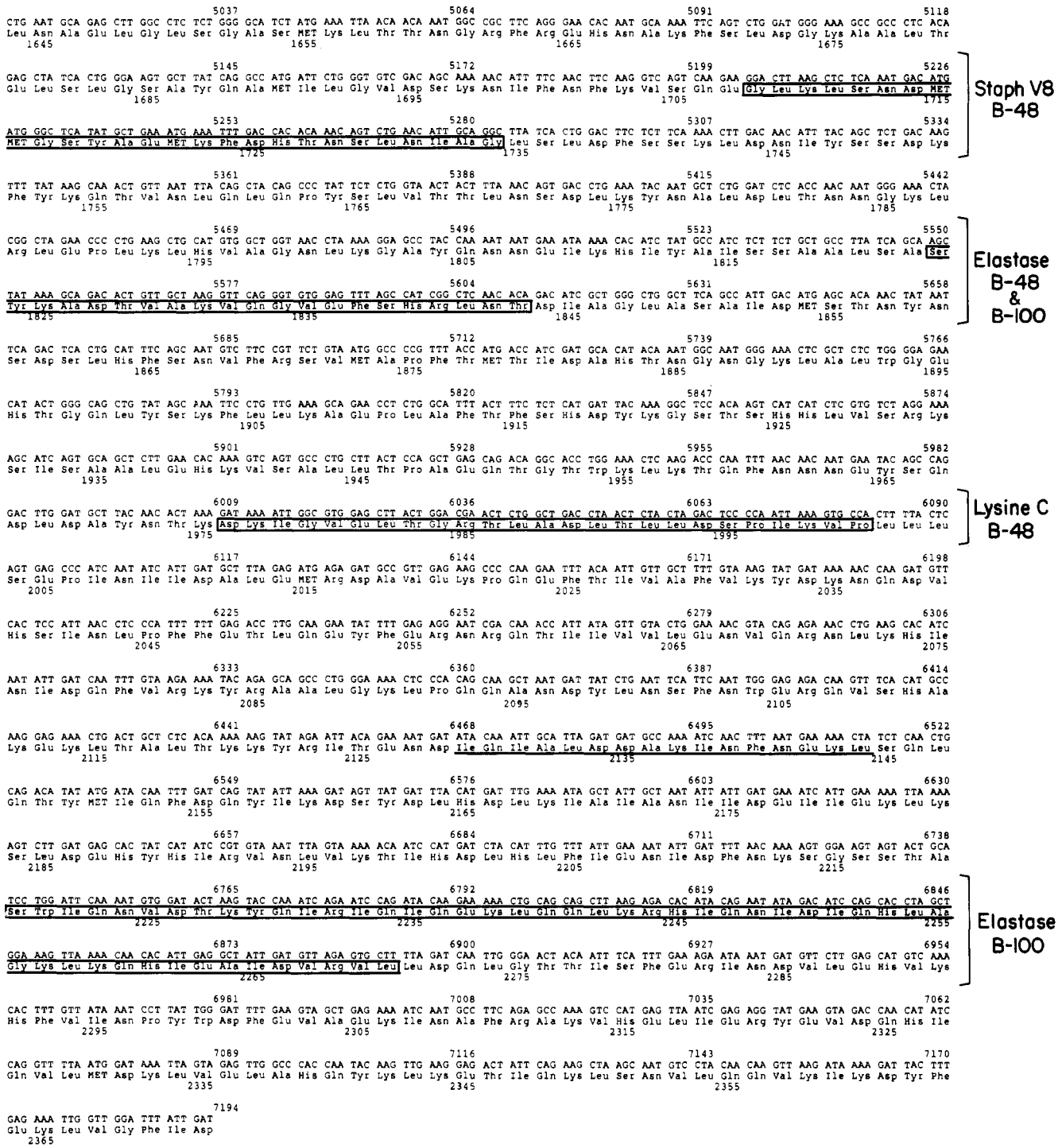

FIGURE 1: Nucleotide sequence and inferred amino acid sequence of apoB cDNA clones. Amino acids that are boxed have been identified by protein sequence analysis. Amino-terminal sequences from peptide B (Staph V8 B-48), peptides C and C* (Elastase B-48 and B-100), and peptide D (Lysine C B-48) are shown. Also shown is the amino-terminal sequence from an additional peptide from an elastase digest of B-100 (Elastase B-100), which does not appear in the elastase digest of B-48. The amino acid sequence of the synthetic peptide corresponding to the carboxyl terminus of B-48 is underlined.

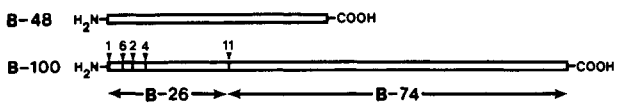

FIGURE 2: Structural orientation of B-48 relative to B-100, on the basis of their identical amino-terminal amino acid sequences. Also shown are the relative positions of synthetic peptides 1, 2, 4, 6, and 11 and of naturally occurring peptides B-26 and B-74, which were used for the production of antisera.

From the silver-stained gels we have also identified at least one prominent band in each digest of B-48 that clearly does not match any band in the parallel digest of B-100. These unmatched bands have been designated A, B, C, and D in Figure 3. To account for the presence of unmatched bands in digests of B-48, it would be reasonable to suggest that B-48 could contain one or more regions that are not homologous with B-100. However, the presence of the unmatched bands does not exclude the possibility that B-48 is homologous, over its entire length, with the amino-terminal portion of B-100, that is, that B-48 is simply a truncated form of B-100. In such a situation, the unmatched bands from B-48 could be the ultimate carboxyl-terminal peptides. Each unmatched B-48 peptide, then, would extend only as far as the carboxyl ter-

Table II: Amino Acid Sequences of Synthetic Peptides Used for Production of Antisera[a]

Peptide #1:	
17 (1,907 daltons)	33
ThrArgPheLysHisLeuArgLysTyrThrTyrAsnTyrGluAlaGluSer	
Peptide #2:	
259 (29,153 daltons)	280
LeuLysLeuGluAspThrProLysIleAsnSerArgPhePheGlyGluGlyThrLysLysMetGly	
Peptide #4:	
399 (44,640 daltons)	415
LeuArgGluIlePheAsnMetAlaArgAspGlnArgSerArgAlaThrLeu	
Peptide #6:	
158 (17,849 daltons)	186
AsnCysSerThrHisPheThrValLysThrArgLysGlyAsnValAlaThrGluIleSerThrGluArgAspLeuGlyGlnCysAsp	
Peptide #11:	
1303 (146,312 daltons)	1325
LeuProSerArgGluPheGlnValProThrPheThrIleProLysLeuTyrGlnLeuGlnValProLeu	

[a] The distance of each peptide from the amino terminus of mature B-100 is shown in amino acid residues and in daltons. Peptides 1, 2, 4, and 6 are located in B-26; peptide 11 begins five amino acid residues downstream from the amino end of B-74.

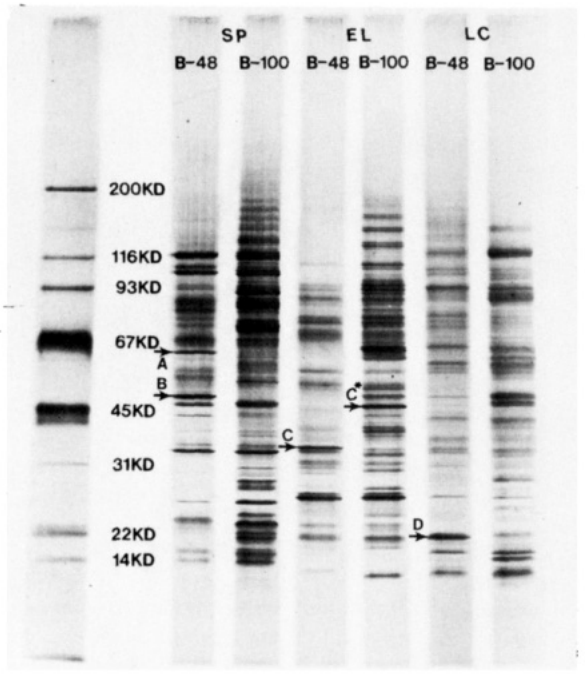

FIGURE 3: Peptides from enzyme digests of B-48 and B-100, separated by SDS–PAGE and stained with silver. Peptides designated A, B, C, C*, and D are described in the text. Standard proteins myosin, β-galactosidase, phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme were used for molecular weight estimations.

minus of B-48, while its counterpart from the longer B-100 molecule would continue on for a distance to the next susceptible enzyme cleavage site, resulting in a mismatched pair. We would always expect to find at least one such mismatch with each different enzyme, unless a susceptible cleavage site in B-100 happened to coincide with the carboxyl terminus of B-48. Lending support to this model is our finding, described in a later section, that all of the unmatched peptides indicated in Figure 3 are derived from the extreme carboxyl end of B-48.

In our experience, silver-stained gels have been most useful in demonstrating the differences between digests of B-48 and B-100, since peptide bands unique to one protein or the other are readily apparent. Less apparent from these gels, however, are peptides representing regions of homology since many bands that are unique to B-100 digests are situated so as to obscure other bands that could be common to digests of both proteins. Thus, to favor the identification of peptides from homologous regions, we have employed the technique of Western blotting, using antisera that recognize selected regions of B-48 and B-100. The resulting patterns reveal a striking degree of homology between B-48 and B-100.

For these experiments, polyclonal antisera recognizing eight regions in B-48 and B-100 were used. Five of the antisera were directed against synthetic peptides that were derived from hepatic and intestinal cDNA sequence. The sequences of the synthetic peptides and their positions within B-100 are shown in Table II and Figure 2. In addition, two antisera were directed againt B-26 and B-74, the two fragments of B-100 that result when low-density lipoproteins are digested with the specific endoprotease kallikrein (Cardin et al., 1984; Hardman et al., 1986). The exact positions of B-26 and B-74 within B-100 are now known (Protter et al., 1986a,b). As is shown in Figure 2, B-26 extends from the amino terminus of mature B-100 for a distance of 1297 residues to an internal cleavage site; B-74 extends from this cleavage site to the carboxyl terminus of B-100. Finally, one antiserum was directed against intact B-48.

All of the antisera against the five synthetic peptides were tested for the ability to recognize intact B-48, B-100, B-26, and B-74 on dot blots and Western blots (data not shown). Each of the antisera recognized both B-48 and B-100, indicating that all of the corresponding peptide sequences are found in both proteins. In addition, antisera 1, 2, 4, and 6 recognized B-26 but not B-74, and antiserum 11 recognized B-74 but not B-26. This pattern of recognition is consistent with the model shown in Figure 2. In these experiments, the strongest reactions were obtained with antisera 1, 2, and 6. These three antisera were then used on Western blots of the enzyme digests.

On Western blots of the enzyme digests, antisera 1, 2, and 6 all yielded similar patterns, indicating that they recognize a similar set of peptides. A typical pattern, with antiserum 2, is shown in Figure 4. From this pattern it is apparent that antiserum 2, like antisera 1 and 6, is able to recognize peptides of several different sizes in each digest. It is most likely that these represent overlap peptides that result from the limited nature of our digests, since a regular pattern of repeating

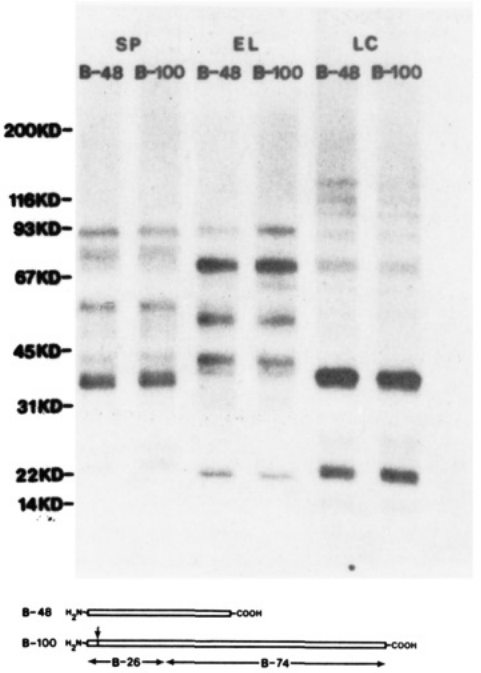

FIGURE 4: Peptides from enzyme digests of B-48 and B-100, separated by SDS–PAGE and subjected to immunoblotting with antiserum against synthetic peptide 2. The region recognized by this antiserum is indicated by an arrow in the inset.

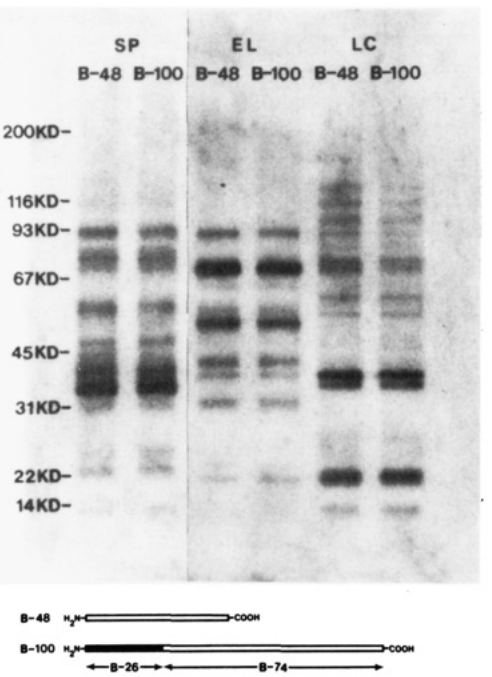

FIGURE 5: Peptides from enzyme digests of B-48 and B-100, separated by SDS–PAGE and subjected to immunoblotting with antiserum against B-26. The region recognized by this antiserum is indicated by a solid bar in the inset.

sequences has not been found in this region of B-100 by cDNA analysis (Protter et al., 1986a,b).

Moreover, with each of the three antisera, the patterns obtained from parallel digests of B-48 and B-100 were virtually identical. Differences were observed only in the largest and smallest peptides and were quantitative in nature, suggesting that there were slight differences in the extent of digestion between parallel digests. In summary, our observations indicate that all of the amino acid sequences recognized by antisera 1, 2, 4, 6, and 11 are found in both B-48 and B-100 and strongly suggest that the disposition of at least three of these sequences (1, 2, and 6) in relation to susceptible proteolytic cleavage sites is identical in both proteins. From these observations, it appears that B-48 and B-100 are extensively, if not totally, homologous in the regions recognized by these antisera.

Next, by use of a polyclonal antiserum against B-26, the patterns obtained from parallel digests of B-48 and B-100 were virtually identical (Figure 5). Since B-26 is a large proteolytic fragment from the amino terminus of B-100, this observation strongly suggests that the amino-terminal regions of B-48 and B-100 are extensively, if not totally, homologous over the entire length of B-26.

Finally, a polyclonal antibody against intact B-48 was used for Western blotting (Figure 6). From the resulting patterns it is apparent that most of the bands recognized by this antiserum are present in digests of both B-48 and B-100. Thus, it appears that the high degree of homology between B-48 and B-100, which was revealed in the preceding experiments, actually extends over most of B-48. However, this antiserum, unlike those used in preceding experiments, was able to recognize additional bands in B-48 digests that are absent from the parallel digests of B-100. Judging from their mobilities on SDS–PAGE, these bands appear to be identical with the unmatched bands from B-48 identified in Figure 3 with silver stain. As was discussed above, the presence of these bands suggests that there could be a region on B-48 that is not homologous with B-100. The fact that these are the only

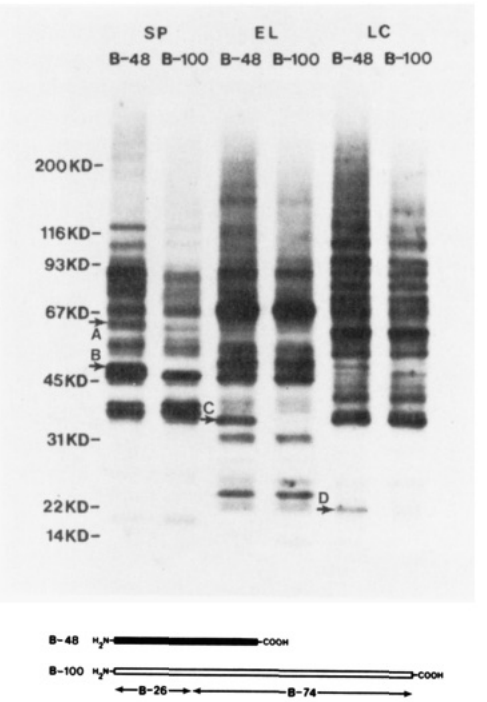

FIGURE 6: Peptides from enzyme digests of B-48 and B-100, separated by SDS–PAGE and subjected to immunoblotting with antiserum againt intact B-48. The region recognized by this antiserum is indicated by a solid bar in the inset. Peptides A, B, C, and D are indicated by arrows on this immunoblot.

unmatched bands that we have observed in B-48 digests suggests that any nonhomologous region, if it exists, is probably associated with these peptides. However, it is also possible that the unmatched bands from B-48 could simply represent the ultimate carboxyl-terminal peptides.

We then tested our polyclonal antiserum against B-48 for the ability to detect regions in B-48 not homologous with B-100. To accomplish this, the antiserum was first pretreated, on a B-100 affinity column, to remove all of the components

that react with B-100. The B-100 on this column was previously denatured with SDS to simulate the denatured state of B-100 on a nitrocellulose blot. Many antibodies in the original antiserum passed through this column without binding to B-100 and were detected on dot blots as described under Materials and Methods (data not shown). The antibodies not retained by the column were then tested for the ability to recognize either B-48 or B-100 (intact or digested) on dot blots and on Western blots. An ability by these pretreated antibodies to recognize B-48 but not B-100 would suggest that B-48 contains a region that is absent from B-100. However, we found no such residual activity in our pretreated antibodies; in fact, pretreatment abolished the ability to react with either B-48 or B-100 (data not shown). In contrast, the same antiserum, when passed through a control column incorporating lysozyme instead of B-100, was indistinguishable from the original untreated antiserum.

We have concluded that all of the regions in B-48 that are recognized by our antiserum againt B-48 are also present in B-100, a finding entirely consistent with our preceding experiments showing extensive homology between the two proteins. It is important to note that the failure of this antiserum to detect a unique region in B-48 does not exclude the possibility that such a region could exist. However, it does suggest that such a region, if it exists, is either not very extensive or not very immunogenic.

In particular, it is apparent that each of the unmatched peptides from B-48 must contain sequence in common with B-100, since components of the antiserum against B-48 that reacted with the unmatched peptides from B-48 also reacted with B-100 on the affinity column. Supporting this conclusion, we have found that all four of the unmatched peptides are recognized by an antiserum against B-74, the carboxyl-terminal proteolytic fragment of B-100 described previously (data not shown). Moreover, according to the model shown in Figure 2, we would expect the amino end of B-74 to overlap the carboxyl end of B-48 for a distance of about 121 000 daltons. Therefore, any peptide from B-48 that reacts with antiserum against B-74 would be expected to arise from this region of overlap, that is, from the carboxyl end of B-48.

Three of the unmatched peptides from B-48 (peptides B, C, and D) were then purified and subjected to amino-terminal amino acid sequence analysis. The resulting sequences were identified in the amino acid sequences derived from both hepatic and intestinal cDNA (Figure 1). The fact that the amino acid sequences from peptides B, C, and D are each coded by hepatic cDNA (coding for B-100) further indicates that the unmatched peptides from B-48 contain sequence in common with B-100.

Since all of the unmatched peptides from B-48 contain sequence in common with B-100, then it is likely that there are counterparts to these peptides in the parallel digests of B-100. In Figure 6, we have found no obvious bands that could represent the counterpart peptides. However, by a process of trial and error, we have identified a single peptide from the EL digest of B-100, designated C* (Figure 3), which is a counterpart to peptide C from the EL digest of B-48. The amino-terminal amino acid sequences of peptides C and C* are identical over the distance analyzed (21 residues), confirming the B-48 and B-100 are totally homologous at this location (Figure 1).

However, despite their demonstrated amino acid sequence homology, peptides C and C* are mismatched with respect to size, peptide C* from B-100 being about 11 000 daltons longer than its B-48 counterpart. As was discussed above, this type of mismatch would be expected if peptide C extended only as far as the carboxyl terminus of B-48 while peptide C*, from B-100, continued on for a distance to the next susceptible enzyme cleavage site.

From the preceding observations, it appears that peptides B, C, and D could actually be the ultimate carboxyl-terminal peptides. To examine this possibility, we have mapped peptides B, C, and D in the known sequence of B-100 and have compared the predicted positions of their carboxyl termini to see how closely they agree. The molecular weights of the peptides were estimated from their mobilities on SDS–PAGE by the method of least squares. Eight proteins ranging in size from 14 400 to 200 000 daltons were used as standards. The estimated molecular masses (and 95% confidence intervals) are as follows: for peptide B, 48 321 (44 982–51 909) daltons; for peptide C, 33 741 (31 205–36 480) daltons; for peptide D, 18 465 (16 527–20 628) daltons. The carboxyl termini of the three peptides map to the following amino acid residues (and 95% confidence intervals) in the apoB sequence: for peptide B, residue 2135 (2107–2164); for peptide C, residue 2120 (2100–2143); for peptide D, residue 2133 (2118–2152).

When compared to one another, the predicted positions of the three carboxyl termini are in such close agreement that they could lie within 20 amino acid residues of one another in the apoB sequence. Considering the expected resolution of this method, it is possible that the three peptides actually terminate at the same position (mean = residue 2130), which is the situation we would expect if they were the ultimate carboxyl-terminal peptides from B-48. This interpretation is supported by the fact that residues 1–2130 encompass about 47% of the B-100 sequence, which is in close agreement with our previous estimate, from SDS–PAGE, that the molecular weight of B-48 is 48% of the molecular weight of B-100 (Kane et al., 1980). Moreover, the calcualted molecular mass of residues 1–2130 is 239 000 daltons, which is in reasonable agreement with our previous estimate, from SDS–PAGE, of 264 000 daltons for the molecular mass of B-48 (Kane et al., 1980), especially considering the reported presence of carbohydrate on the corresponding portion of B-100 (Chen et al., 1986).

Finally, using an antiserum against a synthetic peptide, we have tested B-48 and B-100 for homology at a site predicted to lie at the carboxyl termini of the unmatched peptides. The amino acid sequence of the synthetic peptide corresponds to residues 2129–2144 in the apoB sequence (Figure 1). The resulting antiserum, when tested on dot blots, was able to recognize both B-48 and B-100 (data not shown), indicating that homology between the two proteins extends at least as far as residue 2130 in the apoB sequence. Moreover, this antiserum was also able to recognize peptides A, B, C, D, and C* on Western blots (Figure 7), strongly suggesting that the homology between B-48 and B-100 extends over the entire length of the unmatched peptides from B-48. Thus, even in the unmatched peptides, the only peptides from our digests that are unique to B-48, there appears to be complete homology with a parallel region located near the center of B-100.

It is interesting to note that the antiserum described above (against residues 2129–2144), when used on immunoblots, always gives a visibly more intense reaction with B-48 and B-48 peptides than with B-100 and B-100 peptides, despite the fact that the corresponding sequence has been positively identified in B-100 from LDL by amino acid sequence analysis (Chen et al., 1986). In contrast antisera 1, 2, 4, 6, and 11 reocognize B-48 and B-100 with approximately equal intensity. We have observed this phenomenon on dot blots comparing equimolar

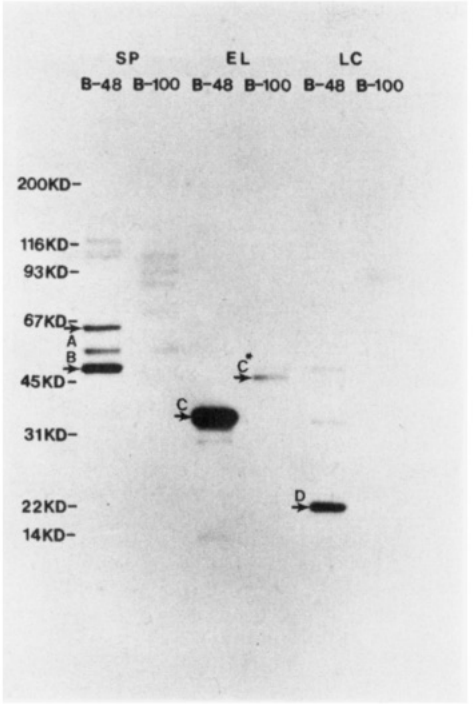

FIGURE 7: Peptides from enzyme digests of B-48 and B-100, separated by SDS–PAGE and subjected to immunoblotting with antiserum against a synthetic peptide whose sequence is predicted to lie at the carboxyl terminus of B-48. Peptides A, B, C, C*, and D are indicated by arrows on this immunoblot.

quantities of intact or digested B-48 and B-100 (data not shown) and on Western blots of peptides separated by SDS–PAGE (Figure 7).

It is possible that the observed difference in reactivity results from a selective posttranslational modification of B-100, such as glycosylation, at the site recognized by the antiserum. However, there is evidence from direct amino acid sequence analysis indicating that B-100 from LDL is not glycosylated or otherwise modified at this site (Chen et al., 1986). Furthermore, because B-100 from both VLDL and LDL react with the same diminished intensity with the antiserum, it appears unlikely that B-100 from either source is modified at this site.

Alternatively, the difference in reactivity observed with this antiserum could be due to a difference in the location of the corresponding sequence relative to the carboxyl termini of B-48 and B-100. In B-48, for example, we have predicted that this sequence is located at the carboxyl terminus, where we would expect a high degree of conformational freedom and accessibility and thus a high probability of recognition by the antiserum. In the longer B-100 molecule, however, this same sequence occupies an internal position (Chen et al., 1986; Knott et al., 1986). As a consequence, it is possible that the site recognized by the antiserum is so constrained or obscured by the continuing peptide chain as to reduce reactivity. We consider this situation to be especially likely in view of the fact that the antiserum was generated with a small synthetic peptide that should possess a high degree of conformational freedom of its own.

In conclusion, it appears that mature, circulating B-48 is homologous over its entire length with the amino-terminal portion of B-100, extending to a point estimated to lie between residues 2130 and 2144 in the B-100 sequence. Thus B-48 contains about 47% of the reported B-100 sequence, which is equivalent to about 240000 daltons of protein. In our digests, the only peptides that are unique to B-48 appear to be totally homologous with a region located near the center of B-100 and do not appear to contain sequence from the carboxyl end of B-100. The extensive homology we have found is consistent with previous evidence suggesting that B-48 and B-100 are coded by the same gene.

However, it is currently unknown how the intestine produces B-48 from a B-100 gene, whether by differential splicing of the B-100 transcript or by posttranslational proteolytic cleavage of B-100. Pulse–chase experiments utilizing human liver and intestine have failed to detect intact B-100 protein in the adult intestine (Glickman et al., 1986), suggesting that proteolytic cleavage of B-100, if it does occur, must be very rapid. Moreover, Northern blot analysis of mRNA coding for apoB has identified two forms in intestine. The most abundant of these comigrates with hepatic mRNA coding for B-100. However, a smaller form is also present that appears to be specific for intestine. This smaller form maps to the 5′-end of the B-100 mRNA and is of sufficient size (approximately 7500 bases) to code for B-48 (Mehrabian et al., 1985; A. A. Protter and K. Y. Sato, unpublished data). However, it is not known whether the smaller mRNA is an artifact that is produced by degradation during isolation or is a native form coding specifically for B-48. From an analysis of the distribution of introns within the B-100 gene, it is not clear how this smaller message would arise by differential splicing of the B-100 transcript, since the predicted site for the carboxyl terminus of B-48 (located near amino acid residue 2140) lies at some distance from the nearest classical introns (located at amino acid residues 1379 and 3903) (Carlsson et al., 1987). Moreover, our analysis of intestinal cDNA clones has found no evidence to suggest that mRNA coding for B-48 differs from that coding for B-100 by virtue of any addition, deletion, or transposition of sequence during mRNA processing. It appears likely, in view of this situation, that B-48 arises as a result of rapid proteolytic cleavage of B-100.

From a functional standpoint, the identity of structure between B-48 and the amino-terminal portion of B-100 implies that this common region plays a common role on triglyceride-rich liploproteins of both intestinal and hepatic origin. It is possible that this region plays a structural role during particle synthesis or secretion, or in subsequent catabolic processes. In contrast, the carboxyl end of B-100, which is absent from B-48, may play a unique role in metabolic processes involving LDL, such as the conversion of VLDL to LDL or the catabolism of LDL. This view is consistent with previous observations indicating that particles containing B-48 do not serve as precursors for the formation of LDL (Malloy et al., 1981; van't Hooft et al., 1982) while particles containing B-100 generally do. This view is also consistent with the recent finding that a receptor binding site, essential for normal LDL catabolism, may be located in the carboxyl half of B-100 (Knott et al., 1985).

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F_o Portion of *Escherichia coli* ATP Synthase: Orientation of Subunit c in the Membrane†

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ABSTRACT: Incubation of right-side-out oriented membrane vesicles of *Escherichia coli* with tetranitromethane resulted in the nitration of tyrosine residues (Tyr-10 and Tyr-73) of subunit c from the ATP synthase. Cleavage of the protein with cyanogen bromide and separation of the resulting fragments, especially of the tyrosine-containing peptides, clearly demonstrated that the distribution of the nitro groups is similar at any time and at any pH value chosen for the analysis. Furthermore, the percentage of 3-nitrotyrosine present in the two peptide fragments was in good agreement with that obtained for the intact polypeptide chain. While the modification of the tyrosine residues in subunit c with the lipophilic tetranitromethane is independent of the orientation of the membrane vesicles, the subsequent partial conversion of the 3-nitrotyrosine to the amino form only occurred when membrane vesicles with right-side-out orientation were treated with the ionic, water-soluble sodium dithionite, which at certain concentrations cannot penetrate biological membranes. Cleavage of subunit c isolated from nitrated and subsequently reduced membrane vesicles and separation of the resulting fragments by high-pressure liquid chromatography showed that the 3-nitrotyrosine in the Tyr-73-containing peptides has been completely reduced, while the nitro group in peptides containing Tyr-10 remained nearly unaffected.

The membrane-bound proton-translocating ATP synthase (F_1F_o) of *Escherichia coli* catalyzes the synthesis of ATP by utilizing energy of an electrochemical proton gradient built up by respiration. The enzyme can also function in the reverse direction by coupling hydrolysis of ATP to proton translocation across the membrane, thereby generating an electrochemical gradient of protons essential for driving, e.g., active transport (Senior & Wise, 1983; Futai & Kanazawa, 1983).

The enzyme complex consists of two structurally and functionally distinct entities designated F_1 and F_o. The water-soluble F_1 part consisting of five different polypeptides (α, β, γ, δ, ϵ) with a stoichiometry of 3:3:1:1:1, respectively, carries the catalytic and regulatory centers of the enzyme (Vignais & Satre, 1984). F_1 is bound by electrostatic and hydrophobic interactions to the membrane-integrated part, F_o, which is built up of three kinds of subunits (a, b, c) and functions as a H^+ channel (Hoppe & Sebald, 1984; Senior, 1985; Walker et al., 1984). The stoichiometry of the F_o subunits is proposed to be 1:2:10 ± 1 for a:b:c, respectively (Foster & Fillingame, 1982). The analysis of mutant strains lacking one of the three F_o subunits (Friedl et al., 1983) and

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