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Direct Cross-Links between Initiation Factors 1, 2, and 3 and Ribosomal Proteins Promoted by 2-Iminothiolane†

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ABSTRACT: Complexes were prepared containing 30S ribosomal subunits from *Escherichia coli* and the three initiation factors IF1, IF2, and IF3. In different experiments, each of the factors was radiolabeled with the others unlabeled. The complexes were allowed to react with 2-iminothiolane and then oxidized to promote the formation of intermolecular disulfide bonds, some of which were between factors and ribosomal proteins. Each of the labeled factors becomes covalently cross-linked to the complex as judged by its failure to dissociate when centrifuged in a sucrose gradient containing a high salt concentration. Proteins from the complexes were extracted and analyzed on two-dimensional polyacrylamide gels by nonequilibrium isoelectric focusing and sodium dodecyl sulfate gel electrophoresis. Spots corresponding to cross-linked dimers that contained initiation factors, as indicated on autoradiographs, were cut out and analyzed further. The following cross-linked dimers between factors and ribosomal proteins were identified: IF1-S12, IF1-S18, IF2-S13, IF3-S7, IF3-S11, IF3-S13, and IF3-S19. Cross-links between factors IF1-IF2 and IF3-IF2 were also identified. A model integrating these findings with others on the protein topography of the ribosome is presented.

Initiation of protein synthesis in bacteria is promoted by three initiation factors: IF1 (M_r 8016), IF2 (M_r 118 000), and IF3 (M_r 20 668). The factors bind to 30S ribosomal subunits during initiation but are absent from 70S ribosomes during elongation [for a review, see Grunberg-Manago (1980)]. We have been interested in defining the ribosomal binding sites for the three initiation factors. The binding of radiolabeled factors to the 30S subunit has been studied by sucrose gradient centrifugation. IF3 binds tightly in the absence of the other components of initiation (Heimark et al., 1976b), whereas IF1 and IF2 bind best in the presence of all three factors (Fakunding et al., 1972; Langberg et al., 1977). The trifactor-ribosomal subunit complex has been postulated to be an intermediate in the initiation pathway (Fakunding et al., 1972; Langberg et al., 1977; Weiel & Hershey, 1981).

Earlier work in this laboratory employed bis(imido esters) to cross-link the three initiation factors to ribosomal proteins (Heimark et al., 1976b; Langberg et al., 1977; Bollen et al., 1975). Antibodies specific for individual ribosomal proteins were used for the identification of constituents in cross-linked complexes that contained initiation factors. Many of the same

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proteins appeared in the cross-links with the different factors. The cross-linked proteins thus identified were IF1 to S1, S12, S13, and S19, IF2 to S1, S2, S11, S12, S13, S14, and S19, and IF3 to S1, S11, S12, S13, and S19. The group thus defined corresponds to a protein neighborhood shown by immunoelectron microscopy to be in the upper one-third and platform part of the subunit (Heimark et al., 1976b; Stöffler & Stöffler-Meilicke, 1981; Lake, 1980) and is consistent with many cross-links between ribosomal proteins in the initiation group (Traut et al., 1980). A major limitation in these experiments was their inability to distinguish ribosomal proteins cross-linked directly to an initiation factor from those cross-linked indirectly through bridges composed of one or more other ribosomal proteins or another factor. The experiments presented here were undertaken to define with greater resolution the binding site for the factors by identifying the proteins cross-linked directly to each one.

The method employed here, reversible cross-linking with 2-iminothiolane, has been used previously to determine the spatial arrangement of proteins in ribosomes and ribosomal subunits (Sommer & Traut, 1976; Kenny & Traut, 1979; Lambert & Traut, 1981). Extensive control experiments showed that the methods developed avoided completely disulfide interchange and possible complications therefrom (Lambert et al., 1978). Cross-linked protein dimers have been analyzed either by diagonal gel electrophoresis or separation by nonequilibrium isoelectric focusing and dodecyl sulfate gel electrophoresis (Boileau et al., 1981). Reductive cleavage of the disulfide-linked dimers and radiolabeling of the monomeric components simplify identification of the monomeric components by electrophoretic analysis.

In the work described here, complexes containing the three initiation factors have been formed. The binding of each factor was quantified in experiments in which it alone was radiolabeled. The complexes containing all three factors were incubated with 2-iminothiolane and cross-linked by oxidation. The proteins, still in the oxidized state and thus containing disulfide-linked dimers, were separated by nonequilibrium isoelectric focusing and dodecyl sulfate gel electrophoresis. Dimers containing ribosomal proteins linked to each initiation factor were located by using the radioactive labels, and their components were identified by reductive cleavage and further two-dimensional gel electrophoretic analysis. Cross-linking between initiation factors was also found.

Experimental Procedures

Urea "ultra pure" and sucrose "RNAse free" were from Schwarz/Mann; 2-iminothiolane was from Serva, and ampholytes were from Bio-Rad; Nonidet P-40 was from British Drug House and Coomassie blue R-250 was from Pierce Chemicals; iodoacetamide and dithiothreitol were from Sigma; acrylamide and *N,N'*-methylenebis(acrylamide) were from Eastman Organic Chemicals and were used without further purification. Triethanolamine was from Eastman and vacuum distilled prior to use. Carrier-free ^{125}I in 0.1 N NaOH was from New England Nuclear and Iodogen was from Pierce.

Preparation of 30S Ribosomal Subunits and Initiation Factors 1, 2, and 3. 30S ribosomal subunits from *Escherichia coli* strain MRE 600 were derived from 70S "tight couples" by zonal centrifugation through a sucrose gradient containing 2 mM $MgCl_2$ as previously described (Boileau et al., 1981). The initiation factors were isolated and purified from *E. coli* strain MRE 600 as described (Hershey et al., 1977). [^{14}C]IF1 and [^{14}C]IF3 were obtained after reductive methylation of the purified factors as previously reported (Heimark et al., 1976b; Langberg et al., 1977). The [^{32}P]IF2 was prepared by phosphorylation of the purified factor with [γ-^{32}P]ATP and rabbit muscle protein kinase (Fakunding et al., 1972).

Formation of Initiation Factor–30S Ribosomal Subunit Complexes and Cross-Linking with 2-Iminothiolane. Three kinds of complexes were prepared, each containing 30S subunits and one radiolabeled and two nonlabeled factors. Previous work (cited above) had established that the labeling procedures had no effect on the activities of the factors. Equimolar amounts of each factor were added to an equimolar amount of heat-activated 30S subunits (0.66 mg/mL) in TEA-SH buffer (50 mM triethanolamine, pH 8.0, 50 mM KCl, 5 mM $MgCl_2$, and 10 mM dithiothreitol). For analytical experiments, 75 pmol of ribosomes and factors was employed, and for gel analysis of cross-linked proteins, 750 pmol was used. The mixtures were heated at 30 °C for 15 min. The radioactive label was used to quantify the binding of each factor by sucrose gradient centrifugation of the small incubation mixtures.

The two large-scale incubation mixtures containing [^{14}C]IF1 and [^{14}C]IF3 complexes were adjusted to 5 mM 2-iminothiolane and incubated for 2.5 h at 0 °C. Under these conditions, the IF2 was cross-linked too extensively and higher multimers predominated over dimers (see below and Figure 2b). Accordingly, the conditions were altered in experiments designed to detect cross-links to IF2. The [^{32}P]IF2 complex was adjusted to 1 mM 2-iminothiolane and incubated for 0.5 h at 0 °C. In all three preparations, disulfide bond formation between the initiation factors and the neighboring ribosomal proteins was promoted by the addition of 40 mM H_2O_2 to the reaction mixtures. The excess of hydrogen peroxide was removed by the addition of 15 μg of catalase/mL of reaction mixture, and the unoxidized sulfhydryl groups were alkylated with iodoacetamide as described previously (Boileau et al., 1981). Aliquots of 0.1 mL were analyzed by centrifugation on sucrose gradients under the conditions described in the legend of Figure 1. The cross-linked and alkylated subunit complexes were extracted in LiCl/urea and acetic acid and lyophilized.

Separation of Initiation Factor–Ribosomal Protein Dimers by Two-Dimensional Gel Electrophoresis. Portions of the lyophilized proteins (0.3–0.4 mg) were resuspended in 40 μL of a solution containing 9.5 M urea, 2% Nonidet P-40, and 2% ampholytes (pH range 3–10) and analyzed by two-dimensional polyacrylamide gel electrophoresis by using a nonequilibrium pH gradient gel in the first dimension and a sodium dodecyl sulfate/polyacrylamide gradient gel in the second dimension (Boileau et al., 1981; O'Farrell et al., 1977). The gels were stained in 0.2% (w/v) Coomassie blue R-250 in methanol/acetic acid/water (5/1/5 v/v) and destained by soaking in several changes of the same solvent. The stained gels were dried on 3MM filter paper and exposed to Kodak No-Screen Medical X-ray film for detection of the radiolabeled initiation factor–ribosomal protein dimers.

Radioiodination and Identification by Two-Dimensional Polyacrylamide/Urea Gel Electrophoresis of Cross-Linked Components Eluted from the Dodecyl Sulfate Slab Gel. Dimer spots that contained radioactive factors were located by superimposition of the autoradiograph on the corresponding stained gel. The spots on dried gels were cut out, extracted in a buffer containing 1% dodecyl sulfate, and iodinated with 0.1 mM KI containing 100 μCi of ^{125}I and Iodogen (Tolan et al., 1980). Variation in the extent of labeling of different proteins is consistent with differences in tyrosine and histidine content; however, all proteins except L7/L12 are iodinated using this procedure. For dimer spots suspected of containing

Table I: Stoichiometry of Binding and Cross-Linking Initiation Factors[a]

	initiation factor–30S subunit (pmol)			
	control		cross-linked	
initiation factor	low salt	high salt	low salt	high salt
[^{14}C]IF1	0.18	0	0.17	0.08
[^{32}P]IF2	0.18	0	0.22	0.03
[^{14}C]IF3	0.22	0	0.19	0.07

[a] Initiation factor–30S subunit complexes were formed and analyzed as described in Figure 1. The amount of each radiolabeled factor bound to the 30S subunit was calculated by summing the amount in the 30S-peak region of the gradient. The amount of 30S ribosomal subunits was determined from the absorbance at 260 nm. One A_{260} unit was considered to contain 75 pmol of 30S ribosomal subunits.

IF2, radioiodination was performed under milder conditions to minimize degradation of that somewhat labile factor. The concentration of iodide and the amount of Iodogen were halved: a reaction time of 1 min was used. In other experiments, all the spots in the regions that contained radioactive factors were cut from undried gels and analyzed. The iodinated proteins, along with 100 μg of total 30S ribosomal proteins used as carried, were dissolved in 50 μL of 8 M urea and submitted to two-dimensional polyacrylamide/urea gel electrophoresis (Kenny et al., 1979). The gels were stained for 30 min with 0.0125% (w/v) Coomassie blue G-250 in 12.5% trichloroacetic acid (TCA) (w/v) and washed twice with 6% (v/v) acetic acid. The gels were dried and radioautographed with Kodak No-Screen Medical X-ray film.

Results

Initiation Factor–30S Ribosomal Subunit Complex Formation. Previous work in this laboratory has characterized the initiation complexes that are formed when the three initiation factors and 30S ribosomal subunits are mixed under appropriate conditions. The complexes are stable at low salt concentration (20 mM NH_4Cl) but dissociate at high salt concentration (1 M NH_4Cl) (Heimark et al., 1976b; Fakunding & Hershey, 1973; Langberg et al., 1977). Acquisition of stability at high salt concentration was used as a criterion of covalent cross-linking of factors to ribosome.

Heat-activated 30S subunits were mixed with initiation factors in the buffer for cross-linking, incubated with 2-iminothiolane, and oxidized. The cross-linked complexes were analyzed by centrifugation in sucrose gradients. Panels a, e, and i of Figure 1 show the binding of [^{14}C]IF1, [^{32}P]IF2, and [^{14}C]IF3, respectively, to the 30S ribosomal subunit at low salt concentration. Quantification of the radioactivity associated with the 30S ribosomal subunit peaks compared to the total added indicates that about 20% of the subunits are involved in the formation of the complex with the factors. The initiation factors 1 and 2 are present in nearly equimolar amounts in the 30S complex (Table I). The amount of [^{14}C]IF3 bound to the subunits is slightly higher. This may result from the fact that IF3 can bind alone to the 30S subunit while the binding of IF1 and IF2 is cooperative and requires the presence of IF3 to achieve maximum and stable binding (Langberg et al., 1977). Centrifugation of the complexes in a sucrose gradient containing 1 M NH_4Cl resulted in the destabilization of the complexes with radioactive factors then appearing at the top of the gradients (Figure 1b,f,j; Table I). Modification of the complexes with 2-iminothiolane and oxidation with hydrogen peroxide (cross-linking) did not alter the interaction between the initiation factors and the 30S subunits (Figure 1c,g,k). However, after cross-linking a portion of each of the bound factors became resistant to dissociation by centrifugation at high salt concentration (Figure 1d,e,h). Incubation with reducing agents prior to centrifugation released the factors (results not shown). The amounts of each bound factor made resistant to release by high salt concentration unless first reduced were 40% of the [^{14}C]IF1, 15% of the [^{32}P]IF2, and 35% of the [^{14}C]IF3 (Table I). The lower amount of cross-linked IF2 is very likely related to the use of 1 mM instead of 5 mM 2-iminothiolane.

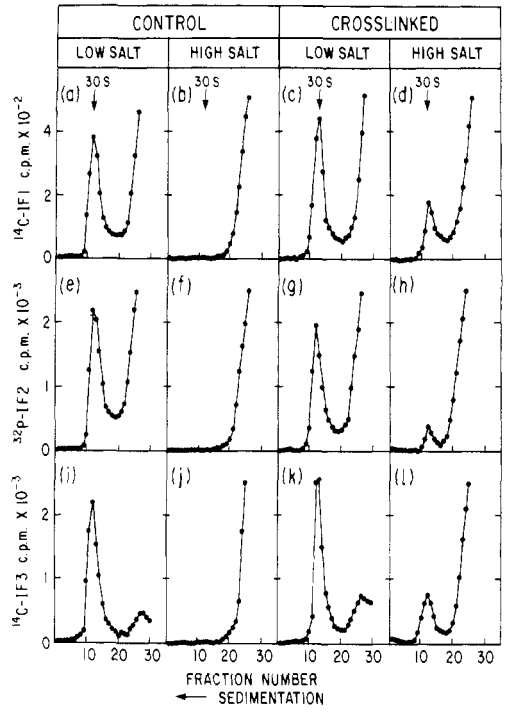

FIGURE 1: Sucrose density gradient centrifugation of initiation factor–30S subunit complexes. Complexes containing [^{14}C]IF1 (sp act. 95 cpm pmol^{-1}) (panels a–d), [^{32}P]IF2 (sp act. 2405 cpm pmol^{-1}) (panels e–h), or [^{14}C]IF3 (sp act. 450 cpm pmol^{-1}) (panels i–l) were prepared as described under Experimental Procedures. Samples (100 μL) of complex, cross-linked or un-cross-linked, were analyzed by sucrose density gradient centrifugation through 7–25% linear gradients in either low-salt buffer [10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (pH 7.5), 20 mM NH_4Cl, 5 mM $MgCl_2$, 6 mM 2-mercaptoethanol] or high-salt buffer [10 mM Tris-HCl (pH 7.5), 1 M NH_4Cl, 5 mM $MgCl_2$]. Gradients were centrifuged at 40 000 rpm for 3.5 h at 4 °C in a Beckman SW56 rotor and fractionated, and radioactivity was determined in toluene/Triton X-100 scintillant. The position of 30S subunits, determined by absorption at 260 nm, is indicated by the labeled arrows. Counts at the top of the gradient do not appear on the scale shown.

Two-Dimensional Electrophoresis of Protein from Cross-Linked Initiation Complexes. The proteins extracted from control and cross-linked complexes were analyzed by two-dimensional polyacrylamide gel electrophoresis by using nonequilibrium pH gradient gel electrophoresis in the first dimension and sodium dodecyl sulfate gel electrophoresis in the second dimension as previously described (Boileau et al., 1981). Figure 2a shows the pattern for the protein mixture extracted from control complexes detected by staining. The position of the three initiation factors and certain ribosomal proteins is indicated.

The gel analysis of cross-linked complexes containing 30S ribosomal subunits and the three factors is shown in Figure 2b. The presence of new species with high molecular weights indicates the formation of intermolecular disulfide bonds. Most of the cross-linked material comes from the 30S subunits, which predominated over initiation factor complexes. The pattern is, however, more complex than that found with 30S

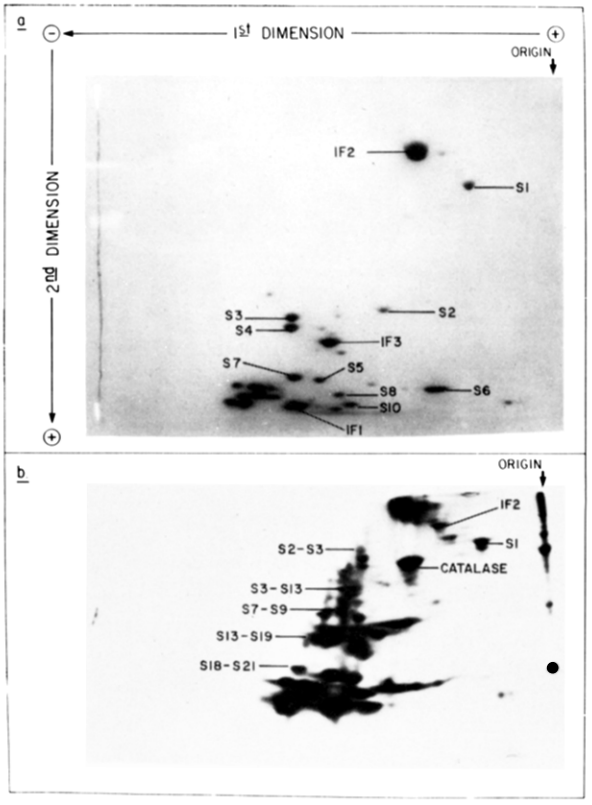

FIGURE 2: Location of initiation factors and reference cross-linked dimers on two-dimensional gel electropherograms. The first dimension was nonequilibrium isoelectric focusing; the second, dodecyl sulfate gel electrophoresis. (a) Mixture of total 30S proteins from non-cross-linked subunits and purified initiation factors. (b) Initiation complexes treated with 5 mM 2-iminothiolane for 2.5 h and oxidized to form cross-links. Known ribosomal protein dimers are marked. Their positions were used to determine the molecular weight calibration scale.

subunits alone (Boileau et al., 1981) due to the presence of additional cross-links involving initiation factors. The ribosomal protein dimers marked in Figure 2b had been characterized previously (Boileau et al., 1981) and were used along with S1 and IF2 to calibrate the second dimension of the gel with respect to molecular weight. The apparent molecular weights of dimers containing initiation factors were obtained from this calibration with known proteins and dimers. Because of the complexity of the pattern, radioactive factors were used in order to locate cross-links containing them.

Identification of Ribosomal Proteins Cross-Linked to IF1. On the autoradiograph of the gel with 30S complexes containing [^{14}C]IF1 (Figure 3a), four labeled spots (A–D) can be seen that contain IF1 dimers with approximate molecular weights of 122 000, 24 000, 22 000, and 17 300 (Table II). The spot to the lower right of D was present in pure iodinated IF1. The position and high molecular weight of spot A strongly suggest that it is a cross-link containing IF1 and IF2. The partner(s) of IF1 in the other spots cannot be deduced so simply.

The spots on the gel corresponding to those lettered in the radioautograph were cut from the gel, and the proteins were extracted, radiolabeled with ^{125}I and reduced. The radiolabeled proteins were then analyzed by two-dimensional polyacrylamide/urea gel electrophoresis in the presence of nonradioactive total 30S ribosomal protein followed by radioautography of the dried gel. Figure 3b shows for reference the positions of all the 30S ribosomal proteins in the gel. The marker proteins in the vicinity of the radioactive component being identified (Figure 3c,d) are indicated by circles, which

Table II: Characterization and Identification of Complexes Containing [^{14}C]IF1

protein complex	M_{r1}[a]	protein pair identified	M_{r2}[b]
A	125 000	IF1–IF2	126 119
B	24 000	IF1–?	
C	22 000	IF1–S12	21 725
D	17 300	IF1–S18	17 017

[a] M_{r1} is the molecular weight of the cross-linked protein complexes estimated from mobility in the second dimension of the two two-dimensional polyacrylamide gel electrophoresis. [b] M_{r2} is the molecular weight of the identified protein pair from the molecular weights of the individual components (Wittmann et al., 1980). The molecular weight of the IF2 used was 118 000 (Hershey et al., 1977).

Table III: Characterization and Identification of Complexes Containing [^{14}C]IF3

protein complex	M_{r1}[a]	protein pair identified	M_{r2}[a]
A	140 000	IF3–IF2	138 693
B	39 000	IF3–S7	37 824
C	34 500	IF3–S11	34 421
D	33 300	IF3–S13	33 661
E	31 500	IF3–S19	30 992

[a] M_{r1} and M_{r2} are as defined in the legend of Table II.

facilitate reference back to Figure 3b. The radiolabeled proteins from spot C are IF1 and S12 (Figure 3c). Protein S18 was found with IF1 in spot D (Figure 3d). The other more diffuse radioactivity visible in Figure 3d did not match any stained marker protein. The molecular weights of the two dimers, IF1-S18 and IF1-S12, calculated from those of the monomers that comprise them (Wittmann et al., 1980) are nearly the same as the values derived from their mobility in the second dimension of the gel (Table II).

Spot B was shown to contain IF1, but no second component could be identified. Likely explanations are that it was inefficiently eluted from the gel or inefficiently iodinated. The molecular weight and its location in the focusing dimension suggest possible candidates. The difference between the apparent molecular weight of the cross-link (24 000) and that of IF1 (8119) is 15 900. Proteins S6, S8, S9, and S11 have molecular weights near this value. Proteins S6 and S8 can be ruled out because they are too acidic to be consistent with the position of B. Protein S11 has been found cross-linked to IF3 (see below). Alternatively, a trimer composed of IF1–S18 and S21 has a molecular weight (25 400) near that of B.

Identification of Ribosomal Proteins Cross-Linked to IF3. Figure 4a shows the radioautograph of the gel containing complexes with [^{14}C]IF3. The five spots (labeled A–E) correspond to protein complexes containing radiolabeled IF3 and have molecular weights of 135 000–140 000, 39 000, 34 500, 33 000, and 31 500 (Table III). The location and molecular weight of spot A shows the presence of IF2 in addition to IF3. The material in spots B through E was extracted, radioiodinated with Na^{125}I and analyzed by two-dimensional polyacrylamide/urea gel electrophoresis. Figure 4b shows the presence of S7 and IF3 in spot B. The circled marker proteins in the immediate neighborhood of S7 and IF3 (left to right, lower to upper) are S8, S5, S4, and S3. In less intense exposures, IF3 is seen clearly distinct from any ribosomal proteins in its vicinity. The apparent molecular weight of complex B is approximately the same as that calculated for the sum of the individual components (Table III).

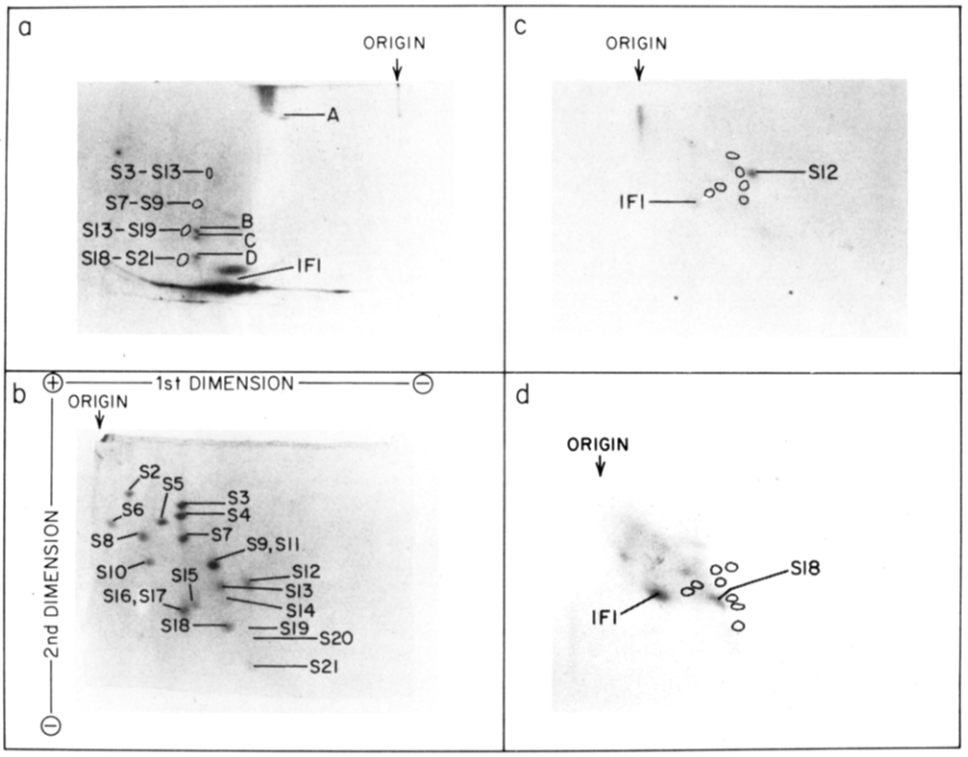

FIGURE 3: Analysis of protein extracted from cross-linked initiation factor–30S subunit complexes containing [^{14}C]IF1. The complexes were modified with 5 mM 2-iminothiolane for 2–5 h and oxidized. Extracted proteins were separated by two-dimensional gel electrophoresis under nonreducing conditions. After being stained and dried, the gel was exposed to X-ray film to produce the radioautograph shown in panel a. Spots labeled A–D correspond to cross-linked protein complexes containing [^{14}C]IF1. The position of stained spots containing known cross-links is indicated. Protein from spots C and D was extracted, radioiodinated, and analyzed by two-dimensional polyacrylamide/urea gel electrophoresis as under Experimental Procedures. The position of all 30S ribosomal proteins, revealed by staining, is shown in panel b. Stained dried gels were then radioautographed to identify the proteins present in spots C and D of panels c and d, respectively.

Spots C–E overlap and could not be cut out without cross contamination. The sensitivity introduced by the radioiodination heightens this difficulty. Three components in addition to IF3 were found in the C–E region. Figure 4C shows the presence of two ribosomal proteins whose identity is clear, namely, S13 and S19. A third component is located at the position for S11 and/or S9. Other diffuse material in the radioautograph did not match the marker proteins. The molecular weight of each of the entities in the region C–E necessitates the presence of IF3 as a component. Dimers between any two of the possible ribosomal proteins (S11, S9, S13, S19) would migrate more rapidly, and trimers would migrate more slowly. Thus the data from Figure 4c and the molecular weight analysis (Table III) demonstrate the presence of cross-links IF3–S13 and IF3–S19 in spots D and E, respectively. In order to resolve the ambiguity concerning the third cross-link, the S11/S9 spot (Figure 4c) was eluted and electrophoresed again in a dodecyl sulfate gel system that resolves S9 from S11(18). Only S11 was found, and the third cross-link in the C–E region is IF3–S11.

Identification of Ribosomal Proteins Cross-Linked to IF2. Proteins extracted from cross-linked complexes containing [^{32}P]IF2 were analyzed by two-dimensional polyacrylamide gel electrophoresis performed in two ways: with nonreducing conditions in both dimensions (Figure 5a) or with nonreducing conditions in the first dimension followed by reduction of disulfide bonds prior to the second dimension (Figure 5b). The distinctive molecular weight of IF2 simplifies the recognition of cross-links related to it and supplements the use of a radioactive factor to achieve this end. There are three such protein complexes visible in Figure 5a, labeled A–C, with approximate molecular weights of 130 000, 125 000, and 135 000, respectively.

Table IV: Characterization and Identification of Complexes Containing IF2

protein complex	M_{r1}[a]	protein pair identified	M_{r2}[a]
A	130 000	IF2–S13	130 968
B	125 000	IF2–IF1	126 119
C	135 000	IF2–IF3	138 693

[a] M_{r1} and M_{r2} are as defined in the legend to Table II.

When the second dimension is run under reducing conditions, the monomeric components of the previously cross-linked protein complexes separate and fall on vertical lines descending from the original position. In Figure 5b (second dimension reduced instead of oxidized) constituents A′ and A″ replace A, and C′ and C″ replace C. (The lightly stained A″ and C″ spots could not be reproduced photographically.) Spot B′ appears only slightly changed from B, and a second spot is not clearly visible below it. Components A′, B′, and C′ migrate with a mobility identical with that of IF2. The identification of ribosomal proteins in A″ and C″ is shown in panels c and d of Figure 5, respectively. Spot A″ is S13, and C″ is IF3. Low-intensity material to the upper left of IF3 does not match the ribosomal proteins (S6, S8) in the vicinity. The original cross-links are therefore IF2-S13 and IF2-IF3. These results were confirmed by analysis of spots A and C from Figure 5a. Spot B could only be characterized by analysis of the still oxidized, cross-linked spot present in Figure 5a. It was shown by radioiodination and two-dimensional electrophoresis to contain IF1 (results not shown). IF2 is cross-linked to both IF3 and to IF1. Table IV shows that the apparent molecular weights of the IF2 cross-links are nearly the same as the sum of the molecular weights of individual components.

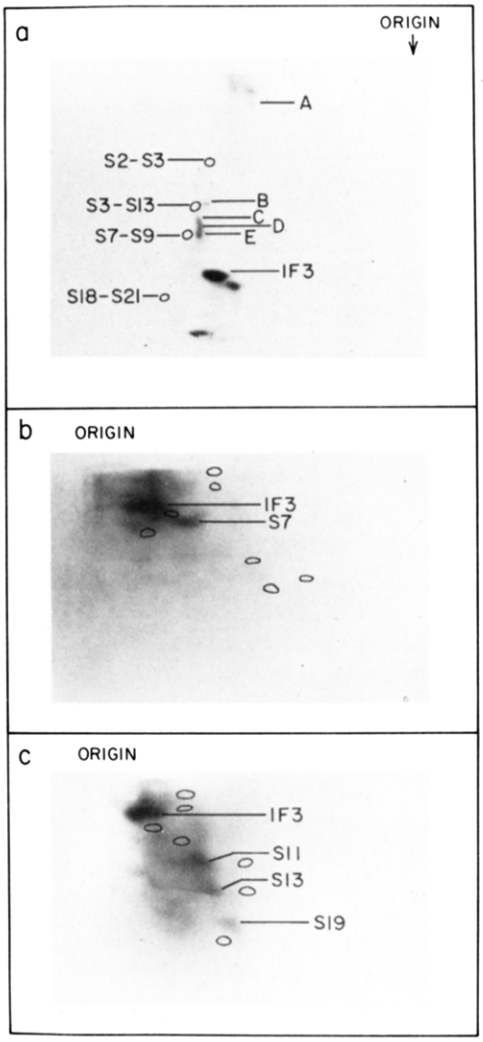

FIGURE 4: Analysis of protein extracted from cross-linked initiation factor–30S subunit complexes containing [^{14}C]IF3. Cross-linked complexes were formed and analyzed as in the legend to Figure 3. Radioautography of the stained dried gel is shown in panel a. Spots A–E correspond to complexes containing [^{14}C]IF3. Identification of the proteins present in spot B and spots C–E is illustrated in panels b and c, respectively.

Discussion

These experiments were undertaken in order to extend and refine previous results from this laboratory (Heimark et al., 1976a,b; Langberg et al., 1977; Bollen et al., 1975) by distinguishing between ribosomal proteins directly cross-linked to each of the three initiation factors and those cross-linked indirectly through an intervening protein bridge. The methods used previously did not permit this distinction to be made.

The three initiation factors 1, 2, and 3 and 30S ribosomal subunits were mixed in equimolar amounts under conditions shown previously to promote the specific binding of the factors to the subunits. Approximately 20% of each of the factors was bound to the 30S subunits, indicating that the factors were bound in equimolar amounts. This stoichiometry, in addition to the dependence for binding IF1 and IF2 on the others factors that was shown previously (Fakunding et al., 1972; Langberg et al., 1977), supports the conclusion that the binding was specific. It is highly likely, although not proven directly, that 20% of the 30S ribosomal subunits formed trifactor complexes. The finding of cross-links between factors supports this interpretation.

The initiation factor complexes were modified by incubation with 2-iminothiolane and oxidatively cross-linked. Two types of evidence demonstrated cross-linking between the factors and the 30S ribosomal subunit: first, the stabilization of the initiation factor–30S complex to centrifugation in high salt concentration; second, the appearance of protein complexes with elevated molecular weights that contained the initiation factors. Identification of the proteins found in these complexes revealed the direct cross-linking of IF1 to the ribosomal proteins S12 and S18, of IF2 to S13, and of IF3 to S7, S11, S13, and S19. Six 30S ribosomal proteins are present in the initiation neighborhood as defined by cross-linking with 2-iminothiolane.

In addition to direct cross-links between ribosomal proteins and initiation factors, cross-links between initiation factors were demonstrated. IF2 was cross-linked to both IF1 and IF3. A thorough search for a cross-link between IF1 and IF3 was made on the gels of cross-linked proteins with both IF1 and IF3 radiolabeled. In neither case could such a cross-link be found.

Diagonal sodium dodecyl sulfate gel electrophoresis has been used extensively for the identification of complexes formed by reversible cross-linking of ribosomal proteins (Sommer & Traut, 1976; Kenny & Traut, 1979; Lambert & Traut, 1981). The systems used in those studies did not resolve well dimers with molecular weights exceeding 60 000. The molecular weight of the IF2 used in this study is 118 000, and cross-links to it would not have been readily identifiable by the earlier methodology. An alternate method was used recently to investigate the cross-links formed by another large ribosomal protein, S1 (Boileau et al., 1981). This technique, which consists of fractionation of total cross-linked protein from ribosomes or initiation complexes by two-dimensional polyacrylamide gel electrophoresis with a nonequilibrium pH gradient gel in the first dimension and a sodium dodecyl sulfate gel in the second (O'Farrell et al., 1977), was used to separate cross-links containing initiation factors.

The binding site(s) on the 30S ribosomal subunit for initiation factors has been investigated by cross-linking methods in this laboratory [Heimark et al., 1976b; Langberg et al., 1977; Bollen et al., 1975) and in others (Hawley et al., 1974; Van Duin et al., 1975; Pon et al., 1977; Cooperman et al., 1977, 1981; MacKeen et al., 1980; Chaires et al., 1982; reviewed in Grunberg-Manago (1980)]. Both bifunctional reagents and UV irradiation have been used. A group of eight ribosomal proteins, S1, S7, S11, S12, S13, S18, S19, and S21, were consistently found cross-linked in significant yield to more than one initiation factor, or to the same factor, by more than one laboratory. The six ribosomal proteins directly cross-linked to initiation factors reported in this paper (S7, S11, S12, S13, S18, S19) are among this group. Cross-links between IF3 and S11, S12, S18, and S21 with a photoactivatable derivative of the factor (Cooperman et al., 1981), to S1, S7, S11, S12, S18, and S19 by direct UV cross-linking of unmodified IF3–ribosome complexes (MacKeen et al., 1980), and to S11 and S12 by disulfide cross-linking of IF3–ribosome complexes containing IF3 previously reacted with 2-iminothiolane (Chaires et al., 1982) have been reported recently. It is likely that the cross-links identified were direct, but the immunochemical methods used to identify the proteins do not absolutely rule out the formation of complexes containing more than two proteins. This study shows unambiguously the direct cross-linking of S7, S11, and S19 to IF3 and adds S13 as a direct cross-link. However, S12 was not among the proteins found here cross-linked to IF3. A cross-link between IF3 and S12 was observed earlier in this laboratory (Heimark et al., 1976b) with dimethyl suberimidate or *N,N-p*-phenylenedi-

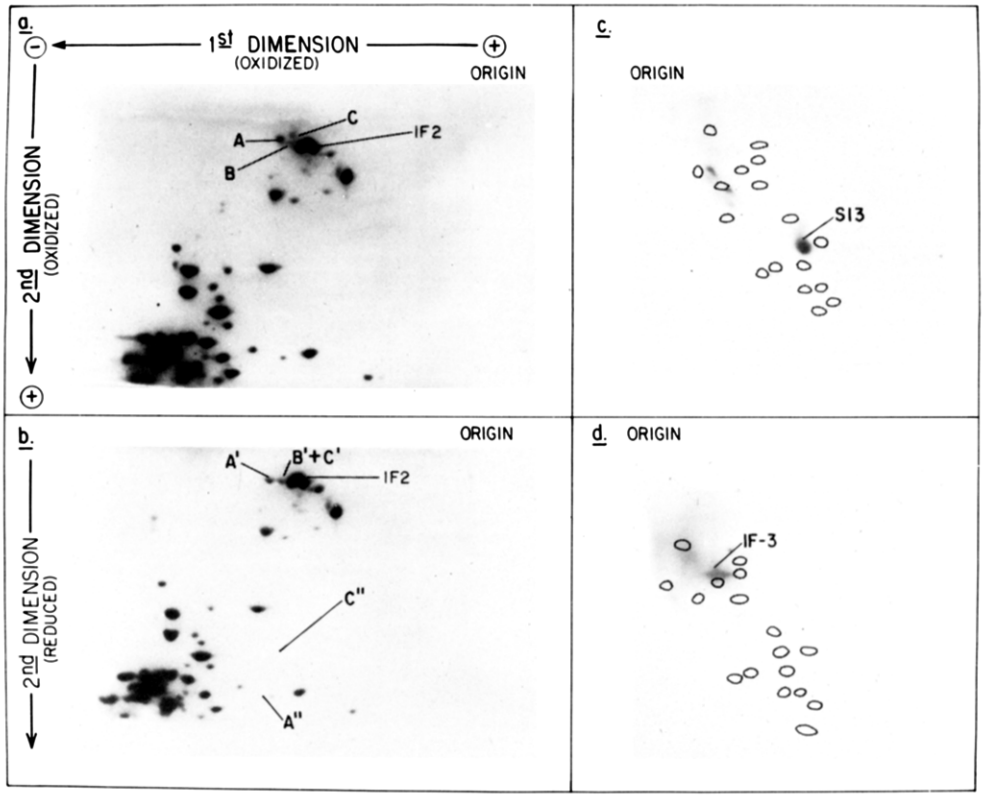

FIGURE 5: Analysis of protein extracted from cross-linked initiation factor–30S subunit complexes containing IF2. The complexes were modified with 1 mM 2-iminothiolane for 0.5 h and oxidized. Extracted proteins were separated by two-dimensional gel electrophoresis under nonreducing conditions (panel a) or under nonreducing conditions in the first dimension followed by reduction of the disulfide bonds prior to electrophoresis in the second dimension (panel b). Spots A–C in panel a (sample oxidized during second dimension) correspond to protein complexes containing IF2. Spots A′, A″, B′, C′, and C″ in panel b (sample reduced during second dimension) correspond to the monomeric components from spots A–C in panel a. Identification of the proteins present in spots A″ and C″ (panels c and d, respectively) was as in the legend to Figure 3.

maleimide although the immunochemical methods used did not show it to be direct. The absence of the IF3-S12 cross-link in this study may be due to the use of a different bifunctional reagent or cross-linking procedure or by the presence of IF1 and IF2 in the complex. There is clear agreement that S12 is located in the initiation region of the 30S subunit since a direct S12–IF1 cross-link was shown here.

The experiments reported here show for the first time that IF1 and IF2 occupy sites that permit the formation of direct cross-links to ribosomal proteins. Although a stable complex between IF1 and the 30S subunit has been reported (Van der Hofsted et al., 1978), binding of IF1 alone appears unstable, and stable binding appears dependent on the simultaneous presence of IF2 and IF3 (Langberg et al., 1977; Hershey et al., 1969; Grunberg-Manago et al., 1973). The present results suggest that ribosomal protein determinants do play a role in the interaction of IF1 with the initiation complex. The two proteins to which IF1 is cross-linked, S12 and S18, do not appear cross-linked to either IF2 or IF3 with 2-iminothiolane.

The cross-linking of the largest factor, IF2, to a single ribosomal protein, S13, is surprising. Careful analysis of the gels of cross-linked proteins with radiolabeled IF2 has not indicated additional cross-linked dimers. This factor plays a role when the initiation complex and 50S subunit join. It is involved in GTP hydrolysis and can be cross-linked to proteins L7/L12 in 70S initiation complexes (Heimark et al., 1976a). This implies the location of IF2, and therefore of the other factors as well, at the subunit interface. A possible explanation for the absence of additional interactions between IF2 and other 30S proteins is its interaction with tRNA and with the 50S subunit, the domains for which are distal to the 30S contact region.

Four of the six proteins defined by these studies as components of the initiation factor neighborhood are at the subunit interface. Proteins S11, S12, S13, and S19 have been identified in cross-links to 50S ribosomal proteins (Lambert & Traut, 1981). Other cross-links have been found between the proteins cross-linked to initiation factors, consistent with their presence in a localized domain. These include S7–S13, S11–S13, S11–S21, S12–S13, and S13–S19 (Traut et al., 1980). A central role or location for IF3 is suggested because it forms cross-links to four of the other five proteins and to two of the factors.

Figure 6 relates the cross-linking data reported here to the asymmetric view of the 30S ribosome model proposed by Lake and the locations assigned to certain proteins on the basis of immune electron microscopy (Lake, 1980). The face of the 30S subunit shown in Figure 6 is thought to be that which contacts the 50S subunit in the 70S ribosome on the basis of electron microscopy (Lake, 1980; Stöffler et al., 1980). This feature of the model has been supported by the identification of cross-linked dimers consisting of one 30S and one 50S protein (Lambert & Traut, 1981). Figure 6A shows the three initiation factors and the 30S ribosomal proteins to which they are directly cross-linked with 2-iminothiolane. The positions shown for S7, S13, S19, and S11 are taken directly from the Lake model. The location for S18 and S21 is inferred from the cross-linked trimer S11–S18–S21 found previously (Shih & Craven, 1973) and from the labeling of S18 and S21 by mRNA and affinity analogues thereof [reviewed by Cooperman (1980)]. The location of S12 is derived from that assigned by Lake (1980) with one important difference. On the basis of immune electron microscopy, Lake finds the location of S12 to be on the surface of the ribosome opposite that shown

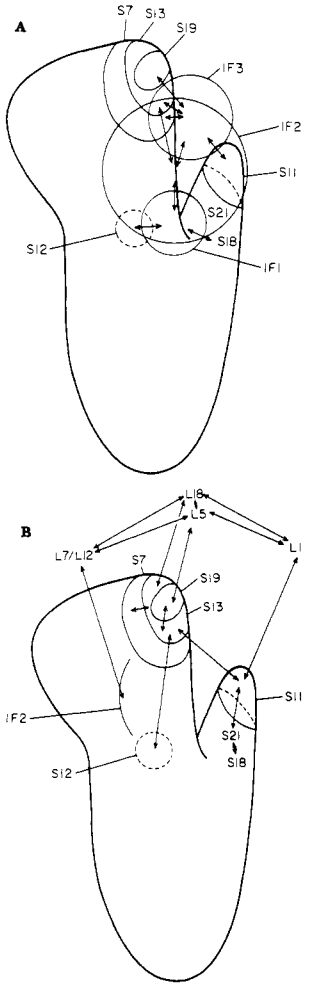

FIGURE 6: (A) Cross-links between initiation factors and 30S proteins. (B) 30S and interface cross-links involving initiation proteins.

here; i.e., away from the interface. Protein S12 is represented as a dotted circle for this reason. The size of the circles representing the initiation factors is proportional to their relative mass and the dimensions of the ribosomal subunit. They are located to give the maximum consistency with data from cross-linking and immunoelectroscopy. Protein IF3 bridges the cleft and is cross-linked to proteins in the head region and in the platform. Protein IF1 is in the neck region, near S12 and S18, overlapping IF2 but distal from IF3 to which it is not cross-linked. Protein IF2 is centrally located between IF1 and IF3 and is apparently spanning the cleft. The absence of additional ribosomal cross-links except S13 and the existence of cross-links to IF1 and IF3 suggest that the latter factors may intervene between IF2 and other proteins in the ribosome. IF1 and IF3 are not obligatory for the binding of IF2 but they may provide part of the functional binding site. IF2, unique among the three factors, has a functionally essential interaction with the 50S subunit. Its location should be at the interface side of the 30S subunit, and it may extend away from that subunit toward the 50S subunit, consistent with a functional role that involves both subunits.

Figure 6B illustrates additional cross-linking data from earlier work (Traut et al., 1980), which reinforces the validity of a 30S subunit model that places the initiation factors and the 30S proteins to which they are cross-linked on the interface side of the 30S particle. IF2 is cross-linked to L7/L12 on the 50S subunit, as well as to S13 (Heimark et al., 1976a,b). Additional 50S cross-links to IF2 were formed but not identified in that earlier study in this laboratory. Proteins S13, S11, and S19, in addition to being cross-linked to IF3, are also cross-linked to the 50S proteins L18, L1, and L5, respectively (Lambert & Traut, 1981). According to models based on immune electron microscopy, L7/L12 and L1 are on the opposite ridge and stalk protuberances of the 50S subunit (Strycharz et al., 1978; Lake, 1980; Dabbs et al., 1981). The 5S RNA and, by inference, proteins L18 and L5, which bind to it, are located in the central protuberance (Shatsky et al., 1980; Stöffler-Meilicke et al., 1981). The interface cross-links S13–L18, S11–L1, and S19–L5 demonstrate the involvement of the three conspicuous asymmetric features of the 50S subunit in interface interactions with the initiation region of 30S, information that contributes to a detailed topographical map of the functional 70S ribosome.

Registry No. 2-Iminothiolane, 6539-14-6.

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Solubilization of Low-Density Lipoprotein with Sodium Deoxycholate and Recombination of Apoprotein B with Dimyristoylphosphatidylcholine†

Mary T. Walsh* and David Atkinson‡

ABSTRACT: Apoprotein B (apoB) of human plasma low-density lipoprotein (LDL) (*d* 1.025–1.050 g/mL) has been solubilized with solid sodium deoxycholate (NaDC) above its critical micellar concentration. ApoB is isolated by gel-filtration chromatography as a mixed micellar complex of protein and detergent in high yield in a lipid-free form. A soluble apoB–dimyristoylphosphatidylcholine (DMPC) complex has been prepared by incubation of aqueous solutions of apoB–NaDC and DMPC–NaDC (2/1 w/w) at room temperature with detergent removal by extensive dialysis. A combination of gel chromatographic and density gradient fractionation of DMPC–apoB incubation mixtures demonstrates that a reasonably well-defined complex of DMPC and apoB is formed with a 4:1 w/w lipid:protein ratio. Negative-stain electron microscopy shows these particles to be single-bilayer phospholipid vesicles with a diameter of 210 ± 20 Å into which the apoB is incorporated. Circular dichroic spectra of NaDC-solubilized apoB show apoB to have similar conformation to that seen in the native LDL particle. However, apoB that has been complexed with DMPC exhibits more α-helix. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis shows a single band (apparent M_r 366 000) for apoB after solubilization, purification, and interaction with phospholipid. The behavior of apoB during its reassociation with phospholipid and the structural features of the DMPC–apoB particle are similar to those observed in the interaction of solubilized membrane proteins with lipid rather than that of other apolipoproteins.

Low-density lipoprotein (LDL)[1] is the primary transport particle for cholesterol in the plasma (Brown & Goldstein, 1976) and is a microemulsion of nonpolar lipids, cholesteryl ester, and triglyceride, whose surface is stabilized by a monolayer of polar phospholipids and cholesterol, together with apoprotein B (apoB) (Nelson, 1979). ApoB functions as the ligand for the LDL receptor in cells of the peripheral tissue

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[1] Abbreviations: LDL, low-density lipoprotein; apoB, apoprotein B; cmc, critical micellar concentration; NaDC, sodium deoxycholate; DMPC, 1,2-dimyristoylphosphatidylcholine; $NaDodSO_4$, sodium dodecyl sulfate; NaPTA, sodium phosphotungstic acid; Tes, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; V_0, void volume; V_t, total volume, L:P, lipid to protein ratio.

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