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Ultrastructure of Native Lipoprotein from *Escherichia coli* Envelopes

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The free form of the major lipoprotein from *Escherichia coli* cell envelopes has been purified to homogeneity by gentle extraction procedures and conventional chromatographic separations in a non-ionic detergent. The morphology of paracrystals obtained from homogeneous protein was investigated by low-dose electron microscopy. Electron diffraction of the paracrystals was consistent with α-helices arranged perpendicularly to the main cross-band with a periodicity of 20 nm.

1. Introduction

The major lipoprotein of Gram-negative bacteria is a small, well-characterized polypeptide consisting of 58 amino acid residues (Braun & Rehn, 1969). There has been considerable interest in this protein since its characterization by Braun (1975). It occurs about $7{\cdot}5 \times 10^5$ copies per cell (De Martini *et al.*, 1976) and expression and processing of its precursors have been studied extensively (Vlasuk *et al.*, 1984). Structurally, it has several unique features: the protein lacks histidine, tryptophan, glycine, proline and phenylalanine. The N-terminal cysteine is linked by a thioether to glycerol, to which two fatty acids are attached by ester linkage and a third is covalently attached to the terminal α-amino group of the cysteine residue Braun & Bosch, 1972). The ε-amino group of the C-terminal lysine is linked to the carboxyl group of every 10th to 12th mesodiaminopimelic acid residue of the peptidoglycan (Braun & Sieglin, 1970). The latter bond occurs in one third of all lipoprotein molecules present. The other two thirds exist in an unlinked or free form (Inouye *et al.*, 1972). It is believed that the lipoprotein plays an important role in stabilizing the cell envelope and in altering the functional properties of various outer membrane and possibly periplasmic proteins by physically interacting with them. A prerequisite for the understanding of these interactions is the knowledge of the native structure of the proteins involved. Former purifications of lipoprotein were based on extracting cell envelopes by boiling them in 4% (w/v) sodium dodecyl sulphate and fractionation with organic solvents. Such treatment is, of course, strongly denaturing, with conversion of the native conformation to a predominantly α-helical structure (Reynolds & Tanford, 1970). The observation that lipoprotein consisted of about 70% α-helices (Braun *et al.*, 1976) therefore required independent evidence. Here we describe a gentle extraction procedure, followed by standard purification techniques. Paracrystals (Inouye *et al.*, 1976; De Martini *et al.*, 1976) of the apparently native form have been studied by electron microscopy. We have also estimated the secondary structure of lipoprotein from our preparation by circular dichroism. The low-resolution structural information has been used to build a model for the molecular interactions of lipoprotein. Structural analysis at higher resolution was performed by applying low-dose electron diffraction techniques to the paracrystals.

2. Materials and Methods

(a) *Culture conditions*

Escherichia coli strain JA221 *lpp*-/F'*lac* i^q/pKEN 125 (Nakamura *et al.*, 1982) was grown in L-broth containing 50 mg ampicillin/l (5×10^8 to 8×10^8 cells/ml). When cell growth reached the end of the exponential

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phase, 0·1 mM-IPTG (isopropyl-β-D-thiogalactopyranoside) was added. After 15 min of incubation, cells were harvested by centrifugation, frozen immediately and stored at −80°C. Approximately 1·4 g of cells (wet weight) were obtained per litre of culture medium.

(b) *Protein determination*

In most experiments, protein concentrations were measured by the method of Bradford (1976). The Biuret reaction was routinely used for protein analysis in the early stages of purification and amino acid analysis for precise determinations.

(c) *Electrophoresis*

Proteins were separated by discontinuous sodium dodecyl sulphate/polyacrylamide gel electrophoresis following the procedure of Laemmli (1970). The samples and standards were run on 15% (w/v) acrylamide gels after denaturation in incubation buffer at 100°C. Protein was stained with Coomassie brilliant blue R250/G250 (4 : 1, w/w).

(d) *Purification procedure*

In a typical purification, 60 g of frozen cell paste was thawed in 230 ml of distilled water containing 1 mM-EDTA. After centrifugation (20 min at 500 ***g***), cells were resuspended in 100 ml of breaking buffer (50 mM-sodium phosphate, pH 7·6) containing 3 mM-NaN_3 0·1 M-NaCl, 5% (w/v) sucrose, 2 mM-$MgCl_2$ and 2 mg each of ribonuclease and deoxyribonuclease. Cells were broken in a French pressure cell. Cell envelopes were pelleted by centrifugation (60 min at 24,000 ***g***) and resuspended in 65 ml of 0·1 M-KP_i (pH 6·6) containing 10 mM-$MgSO_4$ and 2·5 mg each of ribonuclease and deoxyribonuclease. After 30 min of incubation at 37°C, this suspension was centrifuged at 38,000 ***g*** for 30 min. The final membrane fraction was pre-extracted once by suspending the pellet in extraction buffer containing 10 mM-sodium phosphate (pH 5), 5 mM-citric acid, 3% (v/v) octyl-POE (octyl-polydisperse-oligooxyethylene), 3 mM-NaN_3, 0·2 mM-dithiothreitol and immediately centrifuged for 30 min at 37,000 ***g***. Pellets were extracted repeatedly as follows. The cells were resuspended in 60 ml of extraction buffer and incubated for 45 min. The suspension was then centrifuged at 30,000 ***g*** and extractions repeated until no more lipoprotein could be extracted, as judged by polyacrylamide gel electrophoresis. Solubilization was complete after 4 to 6 extractions. The extracts containing most of the lipoprotein were concentrated by pressure dialysis using an Amicon PM-10 membrane. After dialysis against buffer A (25 mM-imidazole · HCl (pH 7), 1% octyl-POE, 0·2 mM-dithiothreitol, 3 mM-NaN_3), the concentrate containing 4 to 5 mg protein/ml was loaded onto a column of Whatman DE52 (5 cm × 25 cm). The bound protein was eluted at 65 ml/h with 1·5 l of a linear gradient from 0 to 0·3 M-NaCl in buffer A. Fractions containing lipoprotein were pooled and concentrated by pressure dialysis as described above. The concentrated solution was dialysed against buffer A and then loaded onto a chromatofocusing column (1·5 cm × 34 cm) pre-equilibrated with buffer A. Lipoprotein was eluted with a polybuffer gradient from pH 7 to 4, which contained 1% octyl-POE and 0·2 mM-dithiothreitol. Lipoprotein-containing fractions were collected, concentrated by pressure dialysis and stored at 4°C. Polybuffer was removed from lipoprotein using a Sephadex G75 column.

(e) *Organic phosphate determination*

The method of Fiske & Subbarow (1925) was used to determine the organic phosphate content of the native lipoprotein.

(f) *Circular dichroism measurements*

Circular dichroism spectra were measured with a CNRS Roussel Jouan Dichrographe III spectropolarimeter. Spectra were analysed by the method of Provencher & Glöckner (1981). The concentrations of the lipoprotein solutions used were determined by amino acid analysis.

(g) *Amino acid analysis and digestion by carboxypeptidase*

Amino acid analysis was carried out with a Durrum D500 set to a sensitivity of 2·5 nmol amino acid. Samples containing approximately 0·1 mg of lipoprotein were hydrolysed in trifluoracetic acid and concentrated HCl (1 : 2, v/v) for 25 min and 50 min, following the procedure of Tsugita & Scheffler (1982). Glycerylcysteine was determined after formic acid oxidation of the lipoprotein (Hirs, 1967). D-Glucosamine content was estimated after hydrolysis in constant boiling (6 M) HCl at 106°C for 24 h in sealed, evacuated tubes. Carboxypeptidase digestion was carried out in pyridine/acetate/collidine buffer (pH 8·5) using DFP (diisopropyl-fluorophosphate)-treated carboxypeptidase A and B (Sigma). Samples were incubated for 16 h at 37°C (Tsugita & van den Broek, 1980).

(h) *Electron microscopy*

Preparations were made by drying the detergent-solubilizing protein (1 mg protein/ml in 1% octyl-POE) in the presence of the stains 1% (w/v) UAc (uranyl acetate) 1% (w/v) PTA (phosphotungstic acid), brought to pH 7·0 with KOH, or 1% (w/v) CaAc (calcium acetate) on carbon/collodion grids. These were examined in a Philips EM301 electron microscope at 80 kV. Images were taken at 25,000-fold magnification, calibrated using negatively stained catalase. Selected area electron diffraction was carried out in the standard conditions with the intermediate lens switched off. The camera length (966 mm) was calibrated against a thallous chloride specimen. Measurements were accurate to within 1%. Low-dose techniques were developed from earlier work (Unwin & Henderson, 1975; Jeng & Chiu, 1983). The dose rates were measured using the optical density of exposed Kodak SO-163 (4463) film developed for 12 min in full strength D19 developed, and using a value of $1e^-/Å^2$ for optical density 2·2. Diffraction patterns were taken with 0·1 to 0·75 $e^-/Å^2$ and images with between 3 and 4 $e^-/Å^2$.

3. Results

(a) *The native state of lipoprotein*

The results of the purification procedure are summarized in Table 1. In a typical preparation, as described in Materials and Methods, 35 mg of pure lipoprotein were obtained from 60 g of wet cells. The lipoprotein was homogeneous as indicated by the single protein band on analytical gel electrophoresis and by comparison of the amino acid composition of the purified lipoprotein with those determined for the bound (Braun, 1975) and the free form (Inouye *et al.*, 1976) of lipoprotein.

Table 1
Purification of lipoprotein

Step	Volume (ml)	Protein concn (mg/ml)	Total (mg)
Crude extract supernatant†	120	9·0	1100
Extracts			
1	65	4·4	286
2	69	2·0	138
3	69	0·35	24
4	61	0·38	23
5	60	0·33	20
6	56	0·26	15
Pooled extracts 2–6	315	0·7	220
DE52 pool	176	0·4	70
Chromatofocusing pool	59	0·6	35

† From 60 g of cells, wet weight.

A significant difference from previous reports was that lipoprotein from our preparation contained one lysine residue less per molecule. Enzymatic degradation with DFP-treated carboxypeptidases A and B revealed that the lipoprotein we isolated lacked the C-terminal lysine that is involved in the linkage to murein in the murein–lipoprotein complex. When treated with carboxypeptidases A and B, the molar ratios of the amino acids released were: Arg, 0·35; Tyr, 0·34; Lys, 0·31 (Nakamura *et al.*, 1980). Carboxypeptidase A alone did not release any amino acid. Preparations were not contaminated with the bound form since diaminopimelic acid and D-glucosamine could not be detected in purified lipoprotein. The phosphate content was less than 0·05 mol of phosphate per mol of the lipoprotein, indicating that the purified protein was essentially free of phospholipids and lipopolysaccharides.

The α-helical content estimated by circular dichroism was 87 (±10)%. This value was measured in the presence of 0·5%, 1% and 2% octyl-POE and 50 mM-sodium phosphate (pH 7·6). An influence of Mg^{2+} on the secondary structure of the lipoprotein, as described by Lee *et al.* (1977), could not be detected.

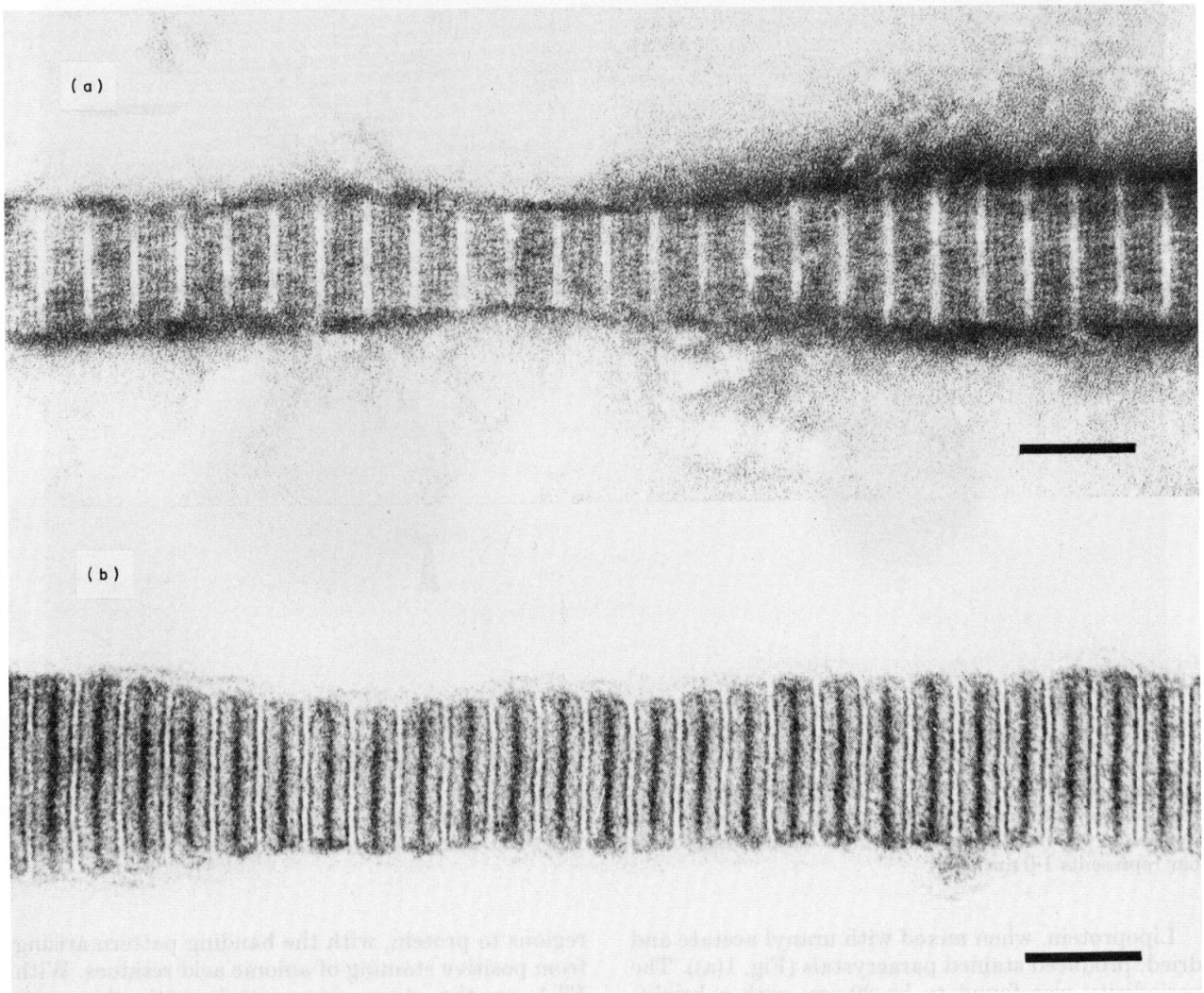

Figure 1. Paracrystals formed by drying in (a) uranyl acetate and (b) potassium phosphotungstate. The scale bars represent 50 nm. Notice the similarity of the overall morphology with those observed previously (De Martini *et al.*, 1976).

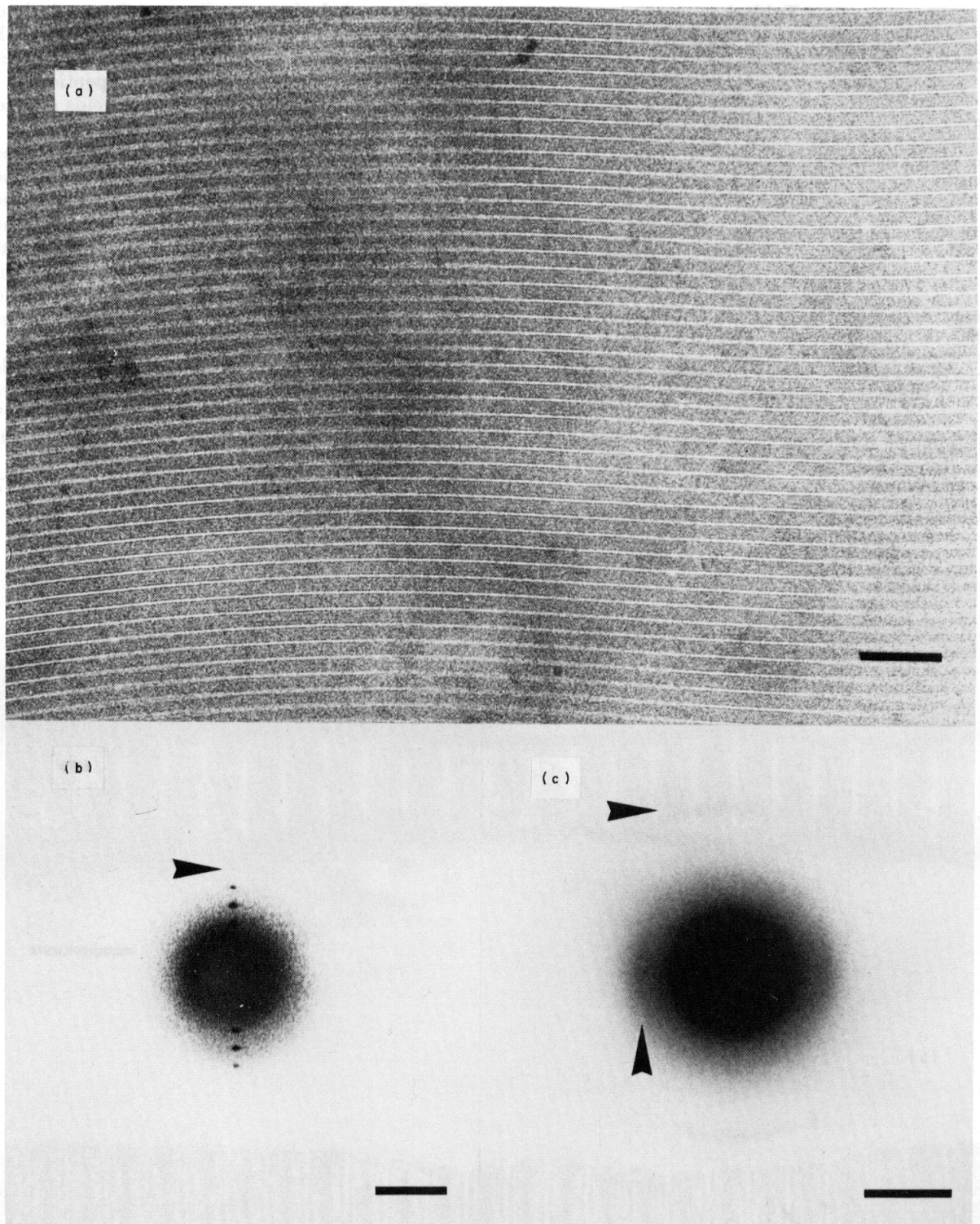

Figure 2. (a) Part of a large paracrystal formed by drying in calcium acetate. The scale bar represents 100 nm. (b) Electron diffraction pattern of a paracrystal as in (a) of a low exposure, optically enlarged so that the scale bar represents 0·2 nm^{-1}. (c) As in (b) but with a longer exposure time and a smaller optical enlargement so that the scale bar represents 1·0 nm^{-1}.

Lipoprotein, when mixed with uranyl acetate and dried, produced stained paracrystals (Fig. 1(a)). The periodicity was found to be 20 nm, with a bright, stain-excluding region 3 nm wide, alternating with a darker banded region of 17 nm width. The light region is likely to correspond to lipid and the darker regions to protein, with the banding pattern arising from positive staining of anionic acid residues. With PTA as the stain, paracrystals with the same periodicity and stain-excluding region were formed but with a different banding pattern reflecting the (positive) staining of cationic residues with the

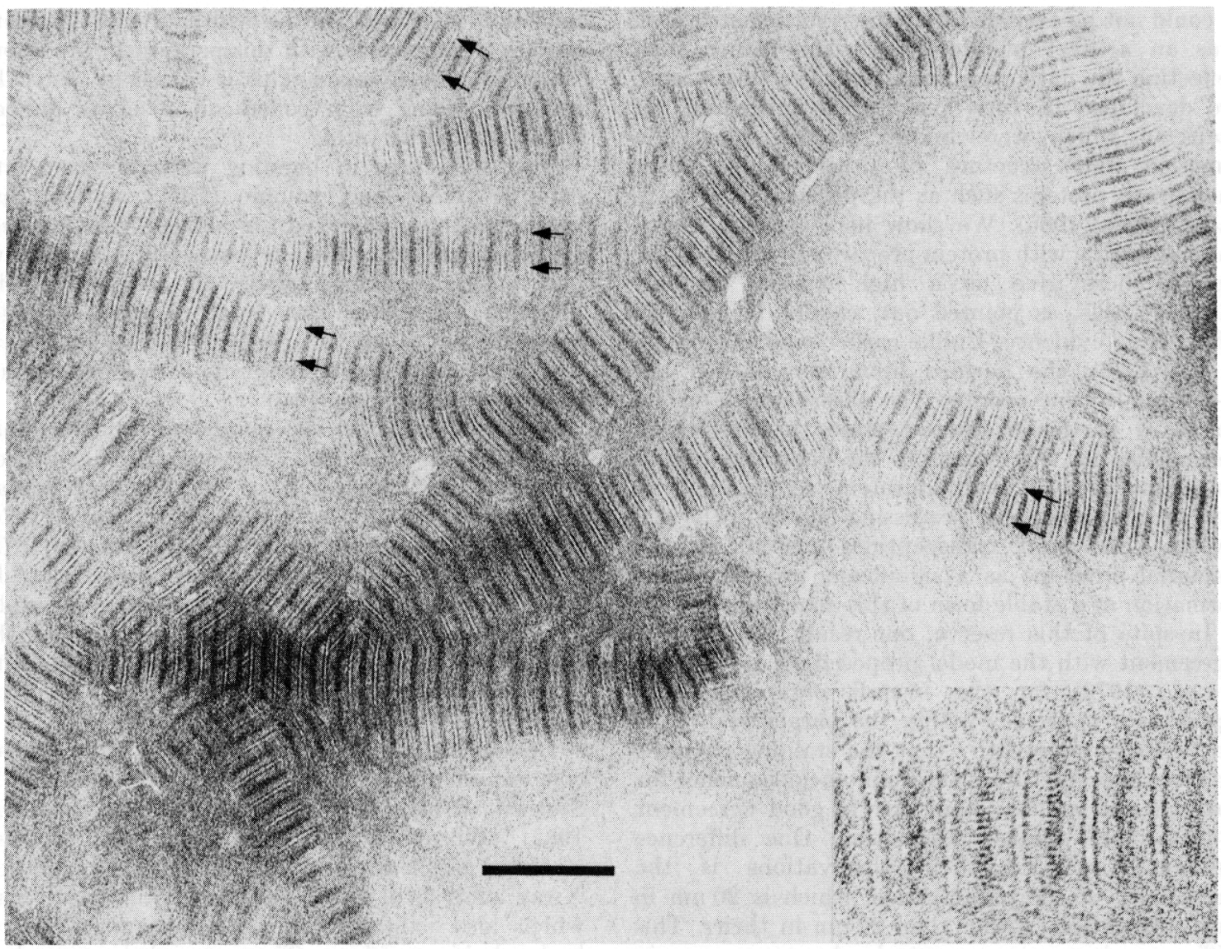

Figure 3. An area of paracrystals formed with a low concentration (0·01%) of PTA and dried with calcium acetate (1%). The arrows point towards regions where the cross-striations are easily seen. The scale bar represents 100 nm. The insertion (bottom right) is at 2·75× higher optical magnification.

anionic dye (Fig. 1(b)). With both UAc and PTA, the banding patterns showed mirror symmetry at right angles to the axis of the paracrystal.

When calcium acetate was used, larger paracrystals up to 5 μm in diameter were formed, which were thin enough for electron microscopy (Fig. 2). These showed the same 20 nm periodicity but banding was less apparent. Electron diffraction from such a paracrystal gave, at low exposure (0·1 $e^-/Å^2$) and 20 nm periodicity extending to the seventh order (Fig. 2(b)). This resolution limit was presumably due to long-range disorder caused by bending of the planes. At an exposure of 0·75 $e^-/Å^2$, the paracrystals gave diffraction patterns showing high-resolution organization (Fig. 2(c)). This pattern is oriented as in Figure 2(b) and shows arcs at 1(±0·1) nm equatorially (perpendicular to the 20 nm spacing of the paracrystal) and arcs at 0·5 nm meridionally (parallel to the 20 nm spacing). These patterns faded with increasing electron dose and had disappeared after 1·0 $e^-/Å^2$.

A double-staining experiment performed on the lipoprotein with PTA at low concentrations (0·01%) did not yield paracrystals on drying. If calcium acetate was present, small and very thin paracrystals formed during drying (Fig. 3). These images again had the overall banding pattern characteristic for PTA staining, but in addition, revealed a 2 nm cross-striation. This fine structure, indicated by arrows in the Figure, was radiation-sensitive (see above) and was only present in the first exposure from any region.

4. Discussion

The only direct indication of structural integrity in proteins that lack measurable biological activity, such as murein–lipoprotein, consists of monitoring their spectral and chemical properties. Combined with structural studies, they may indicate the native conformation and possible changes thereof. Murein–lipoprotein, purified by the procedure of Braun or Inouye and examined by circular dichroism measurements, gave an α-helical content of over 80%. It is known that boiling in dodecyl sulphate tends to cause a shift of proteins into α-helicity (Reynolds & Tanford, 1970) regardless of their native conformation (Tanford, 1973). Since this method was originally used for the purification,

it could not be excluded that the resulting structure was an artifact of the preparation rather than reflecting the native conformation. For this reason, we developed the new purification procedure, all steps of which were known not to affect the function and structure of other *E. coli* outer membrane proteins such as the porins (Garavito & Rosenbusch, 1986). We show here that ellipticity measurements with protein prepared by the method applied here give us a high α-helix content, although this, as pointed out, cannot be used as affirmative evidence. Unlike earlier studies (Inouye *et al.*, 1976), the purified lipoprotein lacked the C-terminal lysine residue. The observation appears to be of particular interest since this residue is responsible for the linkage to the peptidoglycan in the bound form of the lipoprotein. At present it is not clear whether this processing occurs in the cell by the exo- and transpeptides present in the bacterial envelope as a significant process in the formation of a stable form of this structure.

In spite of this reserve, our results are in good agreement with the model proposed by De Martini *et al.* (1976) for the overall organization of lipoprotein molecules within the paracrystal. It is particularly noteworthy that the staining patterns one would predict from the amino acid sequence for cationic and anionic stains are in good agreement with a head-to-head association. One difference between our and their observations is the periodicity of the paracrystals, which is 20 nm in our preparations rather than 22 nm in theirs. This difference does not appear to be due to an error in measurement, but mainly to the thickness of the stain-excluding region (3 nm for our preparation against 4·6 nm in theirs), which presumably consists of lipid and detergent. This could be explained by the different detergents used in the formation of paracrystals. As we have not observed the 0·42 nm acyl chain packing periodicity, typical or a bilayer, and which would be expected on the equator of the electron diffraction pattern, it appears unlikely that the stain-excluding region is a bilayer as has been suggested. A gap of 3 nm is indeed too small to accommodate two palmitoyl chains in extended form in a single bilayer, but could well be due to interdigitation of fatty acyl residues and some detergent.

The 0·52 nm meridional arcs found by electron diffraction are at a spacing typical for coiled-coil arrangements of α-helices (Pauling & Corey, 1953; Cohen & Holmes, 1963), as they have been found in α-keratin (Suzuki *et al.*, 1973), paramyosin (Cohen & Holmes, 1963), tropomyosin (Sodek *et al.*, 1972) and bacteriorhodopsin (Henderson, 1975). The last example is of particular relevance here as the 0·52 nm spacing may be coming from a lipid phase. A 0·15 nm spacing would confirm a protein origin of the meridional reflections. Our failure to observe this is likely to be due to radiation damage that limits the resolution. Tilting has not yet been performed and it may be that curvature of the Ewald sphere was important. The extension of the reflection measured as the width of half intensity implies that features with this periodicity are about 20 nm long on average. This is consistent with the reflection being from coiled-coil α-helices aligned within the paracrystal.

The differences in banding patterns seen with cationic (UAc) and anionic (PTA) staining are similar to those observed for collagen (Doyle *et al.*, 1975) and are related to the distribution of negative and positive charges along the sequence. The presence of a mirror plane in the patterns strongly suggests that there is a regular head-to-head, tail-to-tail arrangement of lipoprotein molecules along the axis of the paracrystal.

A choice exists for the number of α-helices that make up the complex. Two models have been advanced for lipoprotein on the basis of heptad repeats in its amino acid sequence. Inouye (1974) proposed a hexameric form while McLachlan (1978) favoured a dimeric coiled-coil structure. In principle, a triple coiled-coil also seems possible. The 2 nm striations, which we report in the paracrystals shown here, would be compatible with either a double or triple-helix-stranded coiled-coil (Dobb, 1966).

Detailed analyses revealed double helix coiled-coil interactions in tropomyosin (McLachlan & Stewart, 1975), and myosin (McLachlan & Karn, 1982). Wilson *et al.* (1981) described triplet-stranded α-helical coiled-coils in the high-resolution X-ray analysis of haemaglutinin of influenza virus, which also exhibits the heptad arrangement of hydrophobic residues. Three-stranded ropes had been observed earlier in fibrinogen (Doolittle *et al.*, 1978). For the lipoprotein, a firm distinction between these alternatives is not possible on the basis of the diffraction data obtained with dried material.

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References

Bradford, M. M. (1976). *Anal. Biochem.* **72**, 248–254.
Braun, V. (1975). *Biochim. Biophys. Acta*, **415**, 335–377.
Braun, V. & Bosch, V. (1972). *Proc. Nat. Acad. Sci., U.S.A.* **69**, 8154–8159.
Braun, V. & Rehn, K. (1969). *Eur. J. Biochem.* **10**, 426–438.
Braun, V. & Sieglin, H. (1970). *Eur. J. Biochem.* **13**, 336–346.
Braun, V., Rotering, H., Ohm, J. P. & Hagemaier, H. (1976). *Eur. J. Biochem.* **70**, 601–610.
Cohen, C. & Holmes, K. C. (1963). *J. Mol. Biol.* **6**, 423–432.
De Martini, M., Inouye, S. & Inouye, M. (1976). *J. Bacteriol.* **127**, 564–571.
Dobb, M. G. (1966). *J. Ultrastruc. Res.* **14**, 294–299.
Doolittle, R. F., Goldbaum, D. M. & Doolittle, L. R. (1978). *J. Mol. Biol.* **120**, 311–325.
Doyle, B. B., Hukins, D. W. L., Hulmes, D. J. S., Miller,

A. & Woodhead-Galloway, J. (1975). *J. Mol. Biol.* **91**, 79–99.
Fiske, C. H. & Subbarow, Y. (1925). *J. Biol. Chem.* **66**, 375–400.
Garavito, R. M. & Rosenbusch, J. P. (1986). *Methods Enzymol.* **125**, in press.
Henderson, R. (1975). *J. Mol. Biol.* **93**, 123–138.
Hirs, C. M. W. (1967). *Methods Enzymol.* **11**, 197–203.
Inouye, M. (1974). *Proc. Nat. Acad. Sci., U.S.A.* **71**, 2396–2400.
Inouye, M., Shaw, J. & Shen, C. (1972). *J. Biol. Chem.* **247**, 8154–8159.
Inouye, S., Takeischi, K., Lee, N., De Martini, M., Hirushima, A. & Inouye, M. (1976). *J. Bacteriol.* **127**, 555–563.
Jeng, T. W. & Chiu, W. (1983). *J. Mol. Biol.* **164**, 329–346.
Laemmli, U. K. (1970). *Nature (London)*, **227**, 680–685.
Lee, N., Cheng, E. & Inouye, M. (1977). *Biochim. Biophys. Acta*, **465**, 650–656.
McLachlan, A. D. (1978). *J. Mol. Biol.* **122**, 493–506.
McLachlan, A. D. & Karn, J. (1982). *Nature (London)*, **299**, 226–231.
McLachlan, A. D. & Stewart, M. (1975). *J. Mol. Biol.* **98**, 295–304.
Nakamura, K., Pirtle, R. M., Pirtle, I. L., Takaishi, K. & Inouye, M. (1980). *J. Biol. Chem.* **255**, 210–216.
Nakamura, K., Masui, Y. & Inouye, M. (1982). *J. Mol. Appl. Genet.* **1**, 289–299.
Pauling, L. & Corey, R. B. (1953). *Nature (London)*, **171**, 59–61.
Provencher, S. W. & Glöckner, J. (1981). *Biochemistry*, **20**, 33–37.
Reynolds, J. A. & Tanford, C. (1970). *J. Biol. Chem.* **245**, 5161–5165.
Sodek, J., Hodger, R. S., Smillie, L. B. & Turasek, L. (1972). *Proc. Nat. Acad. Sci., U.S.A.* **69**, 3800–3804.
Suzuki, E., Crewther, W. G., Fraser, R. D. B., McRae, T. P. & McKern, N. M. (1973). *J. Mol. Biol.* **73**, 275–278.
Tanford, C. (1973). *The Hydrophobic Effect*, pp. 143–145, John Wiley and Sons, Inc., New York.
Tsugita, A. & Scheffler, J.-J. (1982). *Eur. J. Biochem.* **124**, 585–588.
Tsugita, A. & van den Broek, R. (1980). *Methods in Peptide and Protein Sequence Analysis*, pp. 356–369, Elsevier, North-Holland Biomedical Press.
Unwin, P. N. T. & Henderson, R. (1975). *J. Mol. Biol.* **94**, 425–440.
Vlasuk, G. P., Inouye, S. & Inouye, M. (1984). *J. Biol. Chem.* **259**, 6195–6200.
Wilson, I. A., Skehel, J. J. & Wiley, D. C. (1981). *Nature (London)*, **289**, 366–373.

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