Biochimica et Biophysica Acta, 600 (1980) 72—78

BBA 78853

X-RAY DIFFRACTION AND ELECTRON MICROSCOPY STUDIES OF FROZEN ERYTHROCYTE MEMBRANE PREPARATIONS

L.M. RZEPECKI, J. BERRIMAN * and J.B. FINEAN

Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT (U.K.)

(Received October 31st, 1979)

Key words: X-ray diffraction; Erythrocyte membrane; Freeze-fracture; Glycerol; Freezing

Summary

Well-defined X-ray diffraction patterns have been recorded from erythrocyte membranes in the frozen state. At −40° C, lamellar periodicities range from 19 to 95 nm depending on the glycerol content (0—40%, respectively). Freeze-fracture electron micrographs of samples frozen in two stages to approximate to the diffraction conditions show ice formation external to membrane stacks. The membrane stacks have periodicities of the same order of magnitude as those obtained by X-ray diffraction.

Introduction

Samples of erythrocyte membranes isolated and condensed by ultracentrifugation techniques have been used extensively for X-ray diffraction studies [1—6]. Electron micrographs of thin sections show multilamellar stacks of collapsed membrane sacs, with periodicities of the order of 40—60 nm. Such periodicities are not easily resolved by X-ray diffraction and patterns generally lack definition and detail because of disorder in membrane stacking. Early studies have sought to improve the diffraction characteristics by reducing the periodicity and improving membrane alignment either by controlled partial dehydration or by chemical cross-linking. However, with membranes dried to below 20—30% water content, patterns have indicated a change to a multiphase system [1,2,4]. In these preparations, the periodicity which probably relates to the stacking of membrane sacs is 17—20 nm depending on the source and type of membrane preparation. Lesslauer [5] used phytohaemagglutinin to

* Present address: European Molecular Biology Laboratories, Heidelberg, F.R.G.

stack erythrocyte membranes in the wet state and recorded periodicities ranging from 16 to 18.6 nm. More recently, Pape et al. [6] have recorded the first three diffraction orders of a periodicity of 60 nm from wet erythrocyte membranes prepared in sorbitol, and have calculated an electron density profile of about 2.5 nm resolution for the membranes.

We have recently observed that consistently well-defined diffraction patterns can be obtained from erythrocyte membrane preparations maintained in the frozen state. Diffraction studies have been combined with freeze-fracture electron microscopy and differential thermal analysis in a preliminary study of the freezing conditions required to achieve close-packing of the membranes and of the effects of cryoprotectants such as glycerol.

Materials and Methods

Washed human blood (4—7-days old) from the Midland Blood Transfusion Centre was haemolysed and washed until haemoglobin-free in 20 imosM Tris buffer, pH 7.4, containing 1 mM EDTA (20 imosM EDTA). Where appropriate, up to 40% (v/v) glycerol was incorporated in the final washing buffer. A compact, transparent membrane pellet was formed by centrifugation in the final washing buffer for 2 h at 150 000 × *g*. The inclusion of glycerol tended to make the pellet less compact.

For diffraction studies a sample of the membrane pellet was suspended across the jaws of a stainless-steel holder and sealed in a specimen cell located in the X-ray diffraction camera. The cell temperature could be controlled to ±1°C in the range from —40 to +50°C. Cell humidity could be controlled by aspiration with air bubbled through water or salt solutions. A step-wise cooling rate of 4°C per min was used in most experiments. Samples were used for diffraction either fresh or after slow, partial dehydration at 4°C. A total of about 40 membrane preparations have so far been examined in this way.

A camera with an Elliott toroid focussing mirror was used for medium- to high-angle diffraction recordings, and one of higher resolving power (greater than 50 nm) with single mirror Franks-type focussing for low angles. Both cameras were evacuated during exposure. The X-ray source was an Elliott rotating-anode generator operated at 47 kV, 20 mA. Exposure times varied from 1 to 20 h depending on the resolution required.

Samples for electron microscopy were prepared by fixation in 1% OsO_4 and 1% tannic acid, then dehydrated and embedded in Epon resin. Thin sections were treated with Reynolds' lead citrate stain.

Samples for freeze-fracture were sandwiched between a Balzer's gold 'top-hat' sample holder and a copper ring and either cooled rapidly by plunging them into liquid Freon at —160°C or pre-cooled to —40°C at 4°C per min before rapid cooling in Freon. Samples were fractured in a modified Edwards' freeze-fracture module at —110°C by breaking off the copper ring.

Samples for differential thermal analysis were sealed in aluminium pans and scanned down to —40°C and back to room temperature at speeds up to 8°C/min in a Dupont Differential Thermal Analyser.

Results

Pellets of erythrocyte membranes prepared in 20 imosM EDTA buffer were firm and easy to handle but in the fully hydrated state at room temperature gave diffraction patterns showing only broad maxima in the region of 6.5 and 10 nm [4]. When such samples were cooled, the low-angle diffraction patterns were observed to change abruptly at temperatures between 0 and −10° C, depending on the degree of supercooling. The change in diffraction coincided with the formation of ice as determined by visual inspection of the sample or by detection of the hexagonal ice diffraction pattern. The diffraction pattern of the frozen sample featured three diffraction orders relating to a periodicity which reduced to 19–20 nm at −33° C (Fig. 1a). When samples were re-warmed, this characteristic 'frozen' pattern persisted beyond the initial freezing temperature and the periodicity increased more rapidly as the temperature approached 0° C, but it also became more diffuse and difficult to measure accurately. At about −5° C, an additional minor periodicity was observed which increased from 5.8 to 7.1 nm at −1° C, but which was no longer detected above 0° C.

One of the membrane preparations used in this series of experiments yielded unusually well-defined low-angle diffraction even at room temperature and this was used to obtain a complete sequence of patterns (at 5° C intervals) through the freeze-thaw cycle. These results are presented in Fig. 2 and provide the only reliable periodicity measurements that were obtained above 0° C. The 1200 Å periodicity was deduced from well-defined second- and third-order diffractions recorded in five successive patterns down to −10° C, and again on thawing.

The diffraction periodicities of glycerol-treated membrane samples cooled to −40° C increased linearly with increasing glycerol concentration (Fig. 3). At 40% glycerol, the periodicity at −40° C was increased to 95 nm. Most membrane samples prepared in the presence of glycerol required partial dehydration before freezing in order to obtain a well-defined diffraction pattern, but the periodicity obtained appeared to be independent of the extent of dehydration

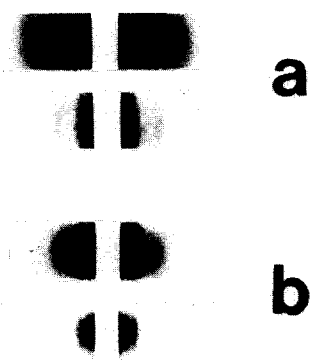
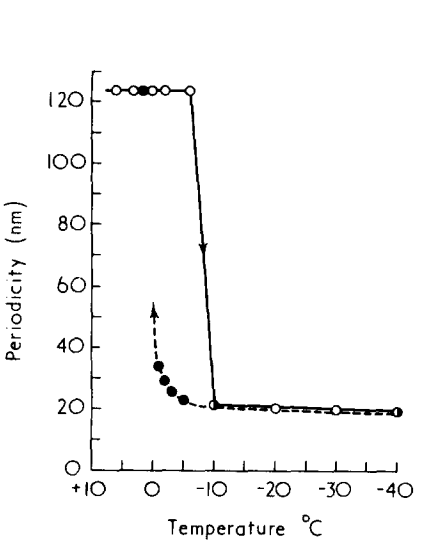


Fig. 1. X-ray diffraction patterns of frozen erythrocyte membrane pellets at −33°C. (a) 20 imosM EDTA preparation. Periodicity is 19.9 nm. (b) 20 imosM EDTA preparation incorporating 20% glycerol. Peridiocity of 55.9 nm was derived from 2nd and 3rd orders. The sample-to-film distance was 218 nm. Exposure time was 20 h.



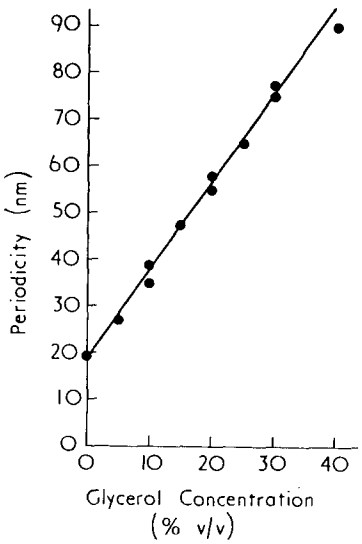


Fig. 2. Changes in periodicity with temperature of an erythrocyte membrane pellet. (○) cooling scan, (●) warming scan.

Fig. 3. Changes in periodicity with glycerol concentration (see Materials and Methods) in erythrocyte membrane preparations maintained at —40°C.

before freezing. In these preparations, 5th- and 6th-order diffractions were observed in addition to the first three (Fig. 1b) and they were appreciably sharper than those obtained from glycerol-free membranes in the frozen state.

Phase contrast microscopy of erythrocyte ghost suspensions exposed to 20% glycerol (or similarly to glycol, sorbitol, sucrose, dextran, etc.,) showed the ghosts to be completely collapsed. Cytoplasmic surfaces appeared very closely apposed.

Electron micrographs of thin sections of membrane pellets (Fig. 4a) show extensive alignment of collapsed ghosts, with a periodicity of 45—55 nm. The cytoplasmic separation is granular while that between membrane sacs is both smaller and less dense.

Electron micrographs of freeze-fractured membrane pellets in 20% glycerol prepared by rapid freezing from about 5°C (Fig. 4b) show extensive regions of cross-fractured membranes, with a periodicity of 85—100 nm. Samples freeze-fractured without pre-treatment with glycerol gave essentially similar but less well-defined images. Membrane pellets pre-frozen to —40°C before further rapid cooling to —190°C (Fig. 4c) show large ice crystals with regions of condensed membrane in between. In such samples, the membrane periodicity is 45—50 nm which is close to that measured by X-ray diffraction studies of equivalent samples (Fig. 1b). In freeze-fracture micrographs of samples subjected to either freezing procedure, the cytoplasmic and external faces of the membranes could be distinguished by identifying either a complete ghost or alternating particle-rich and particle-poor A and B membrane fracture faces (Fig. 4d). As in thin sections, the cytoplasmic separation appeared both wider and more dense than the external separation.

Differential thermal analysis of membrane pellets during cooling indicated

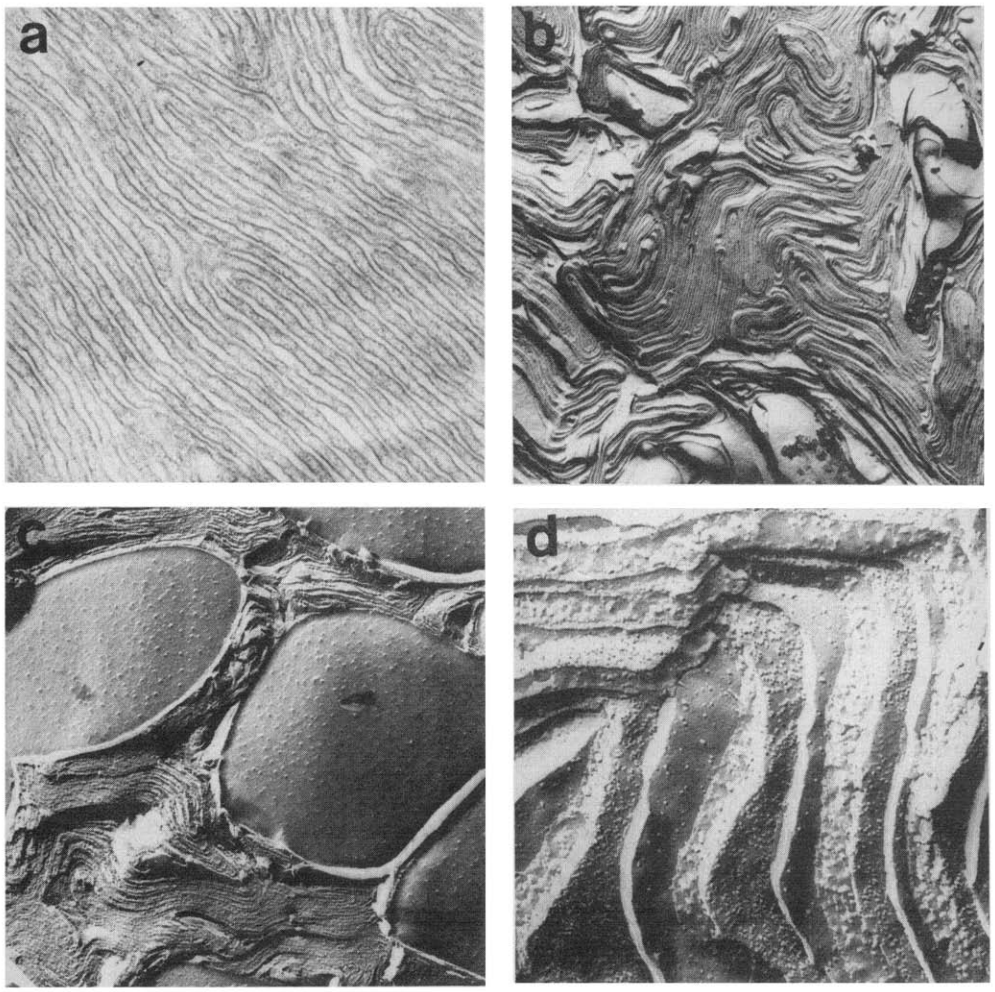


Fig. 4. Electron micrographs of erythrocyte membrane pellets. Membranes for freeze-fracture contained 20% glycerol. (a) Thin section of OsO_4 and tannic acid fixed 20 imosM EDTA membrane pellet (×50 400). (b) Replica of freeze-fractured 20 imosM EDTA membrane pellet cooled rapidly from 25 to −196°C (×8060). (c) Replica of freeze-fractured 20 imosM EDTA membrane pellet cooled slowly to −40°C, then rapidly to −196°C (×21 800). (d) Replica of freeze-fractured 20 imosM EDTA membrane pellet cooled rapidly from 25 to −196°C (×63 800).

extensive supercooling which was increased in the presence of glycerol. Both cooling and warming scans closely resembled those of the equivalent glycerol/water mixtures. Contrary to expectations [7], the effects of the membranes themselves on the cooling characteristics of the whole system appeared small.

Discussion

It is clear from the present X-ray diffraction and electron microscope studies that ultracentrifugation of our 20 imosM EDTA erythrocyte ghost preparation produces extensive arrays of stacked membrane sacs with periodicities of the

order of 100 nm. This periodicity is preserved in the standard freeze-fracture procedure but is considerably reduced by the preparative techniques required for thin-sectioning. This observation is consistent with the general conclusion that the high periodicity includes a large contribution from predominantly aqueous layers which can be substantially reduced by dehydration.

In previous X-ray diffraction studies of partially dehydrated membrane systems, the point at which dehydration causes modification of membrane structure has been identified with a change from a single- to a multi-phase diffracting system [1,2,4]. The frozen membrane periodicity is a little higher than the major periodicity of air-dried membranes but it remains a single-phase diffracting system. The additional minor periodicity, which could relate to a separate lipid phase, was observed only during thawing. It seems probable, therefore, that the frozen membranes retain water essential to their structural integrity, perhaps that water identified by differential thermal analysis as non-freezable water [8], and that the process of slow freezing offers a consistent method for reducing water content of membrane systems to a minimum without causing their breakdown.

From the freeze-fracture electron micrographs (Fig. 4b and c) it is clear that although the overall layer periodicity is markedly reduced by the slow pre-freezing to −40° C the ratio of internal to external surface separations is little changed. This would suggest that the freezing process removes water from both internal and external separations in similar proportions.

Resolution at higher orders in the diffraction patterns so far obtained from glycerol-free samples is probably limited by the smallness of the condensed membrane regions. The improved X-ray patterns from frozen, glycerol-treated membranes, after partial dehydration, may be due to the formation of smaller ice crystals.

The observed linear relationship between the initial glycerol content of the pellet and the diffraction periodicity in the frozen sample is readily explained by reference to the glycerol/water temperature phase diagram [9]. Starting from a glycerol concentration lower than that of the glycerol/water 'eutectic', cooling will lead to the separation of ice crystals which will continue until the glycerol concentration of the residual liquid glycerol/water mixture has increased to the equilibrium value for the selected temperature. At −40° C, the residual liquid phase is about 65% glycerol, and the amount of water removed as ice in order to reach this glycerol concentration is inversely related to the starting concentration. Thus, the higher the initial glycerol concentration, the smaller will be the amount of water removed from the membrane stacks and so the periodicity will be increased accordingly. The slow rate of cooling ensures that ice crystals grow outside the membrane stacks and extract water from them. The much higher membrane periodicity seen in freeze-fracture electron micrographs of rapidly frozen samples indicates that under those conditions the system becomes solidified without appreciable water migration.

Acknowledgements

We are grateful to the Science Research Council for financial support and to Cadbury Ltd., Bournville, for the use of the Differential Thermal Analyser.

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