Reversible Transformation of Precipitated and Nonprecipitated Lipoproteins Recombined from Proteins and Lipids of Erythrocyte Membranes

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The sedimentation at low speed centrifugation of a lipoprotein recombined from the lipids and the strongly bound proteins of the human erythrocyte membrane depends on pH: between 4.5 and 6.0, most of the liproprotein sediments, whereas at pH 7.0–8.5, up to 90% remains in the supernatant. Precipitation of the lipoprotein can be reversed by increasing the pH, followed by a brief sonication. The mobility of spin-labelled protein groups in the lipoprotein increases with increasing pH. This mobility increase is also reversible and is of equal magnitude in precipitated and nonprecipitated recombinates. It is concluded that, because of these reversibilities, determination of the yield of liproprotein formation in recombination experiments must include analysis of both precipitated and nonprecipitated lipoproteins.

Introduction

In experiments on the association of membrane proteins and lipids *in vitro*, the appearance of a precipitate has sometimes been regarded as a criterion for lipoprotein formation. In one case, this criterion was indeed shown to yield results identical to those from equilibrium density gradient centrifugation [1]. Recently, it was also applied by Barzilay *et al.* [2]. These authors, studying the recombination of proteins and lipids from erythrocyte membranes, have used the amount of precipitating material (defined as the amount of material which could be collected by a centrifugation at $20000 \times g$ for 15 min) as a quantitative measure of the yield of the recombination process. Nonprecipitated material was regarded as being of no relevance with respect to the problem of protein-lipid association. From the results of their experiments which showed a pronounced decrease of the yield with increasing pH, Barzilay *et al.* arrived at conclusions on the binding forces governing the association process which differ markedly from the conclusions drawn by our group [3–5]. On the other hand, in our recombination experiments on proteins and lipids from erythrocyte membranes, both precipitated and nonprecipitated lipoproteins were obtained, depending on the conditions of recombination [3–5]. This seemed to indicate that the tendency to precipitate is not an inherent property of a recombined lipoprotein but only a secondary effect which may or may not accompany recombination. To settle this question, we have performed two kinds of experiments: 1. studies on the effect of pH on the sedimentation properties of an isolated lipoprotein, and 2. studies on the effect of pH during and after recombination on the electron spin resonance (ESR) signals of spin-labelled recombinates. These experiments will be described in this paper.

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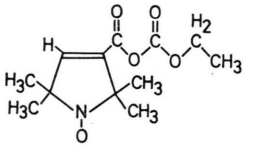
Materials and Methods

Isolation of recombined lipoproteins

The starting materials for the preparation of recombinates: the "strongly bound" protein fraction from human erythrocyte membranes and liposomes from total lipids of erythrocyte membranes, were prepared as described earlier [3, 4]. Recombination was performed by mixing the protein solution (pH approx. 9) with the liposomes and dialyzing the mixture against buffers of the desired composition and pH. Separation of the recombined lipoproteins from uncombined protein and lipid was done by equilibrium sucrose density gradient centrifugation (rotor SW 27, Spinco L2-50B centrifuge) [3, 4]. The

samples were then freed from sucrose by dialysis against recombination buffer.

For the ESR studies, before recombination the proteins were covalently spin-labelled with a pyrrolin nitroxide first described by Griffith *et al.* [6]:



The preparation of the label and the labelling procedure were performed according to ref. 6, using 1 mg label per 20 mg of membrane protein (pH 8.0). Unbound nitroxide was removed from the protein samples by prolonged dialysis against water adjusted to pH 8.

Sedimentation studies

The lipoproteins used for these studies were prepared as described above, the dialysis buffer being 100 mM NaCl, 20 mM acetic acid/NaOH (pH 5.5), 0.5 mM EDTA. The protein/lipid weight ratio was approx. 1.0. After gradient centrifugation and removal of the sucrose by dialysis, the material was sedimented by low speed centrifugation and then resuspended in STE buffer (100 mM NaCl, 10 mM Tris-maleate/NaOH, 0.5 mM EDTA), pH 8.5, at a protein concentration of approx. 2.0 mg/ml. A brief sonication (10 sec at a nominal output power of 50 mW, Branson sonifier B-12) was applied to obtain homogeneous dispersions. Afterwards, the sample was divided into fractions of 2 ml, and the fractions were dialyzed overnight against STE buffer of pH 4.5, 5.0, ... 8.5. The samples were then centrifuged for 15 min at $20000 \times g_{av}$ (13500 rpm in the rotor SS 34, Sorvall RC 2-B centrifuge). Following centrifugation, pellet and supernatant were separated from each other and, after solubilization of the pellet by addition of 1% sodium dodecyl sulphate and removal of the buffer by dialysis against water, were assayed for protein [7] and phospholipid [8] content.

To ensure that the treatment at pH 8.5 did not lead to a decomposition of the recombined lipoproteins, one of the pH 8.5-samples was subjected to equilibrium density gradient centrifugation in a sucrose gradient (0.10 – 0.70 g sucrose per ml in STE buffer, pH 8.5). Centrifugation was performed for 20 h at $240\,000 \times g$ (rotor SW 50.1, Spinco L2-50B ultracentrifuge).

ESR studies

Recombinates containing spin-labelled protein were prepared by dialyzing protein-liposome mixtures against 100 mM NaCl plus 20 mM acetic acid/NaOH, pH 5.5, or 20 mM Tris/HCl, pH 7.3 and pH 8.5. The protein/lipid weight ratio in the mixtures was approx. 1.0. After gradient centrifugation and removal of the sucrose, aliquots of each sample were dialyzed against the buffers of the other samples. In addition, in aliquots of the samples brought to other pH values, pH was afterwards reversed to the starting value. Then, the ESR spectra of all samples were measured in a Varian E-9 spectrometer equipped with variable temperature accessory (E-257). Microwave frequency was 9.5 GHz. During the measurements, the temperature of the samples was kept at 20 °C.

Results

Sedimentation studies

Lipoproteins from liposomes and the strongly bound protein fraction of erythrocyte membranes, when recombined at pH 5.5, flocculate and can be collected by low speed centrifugation [3, 4]. However, if these lipoproteins are brought to pH 8.5, they can easily be converted into an nonparticulate form by a brief sonication. The opalescent solutions thus obtained were dialyzed against buffers of various pH and then subjected to a centrifugation under the conditions used by Barzilay *et al.* for the collection of recombined lipoproteins [2]. Afterwards, the distri-

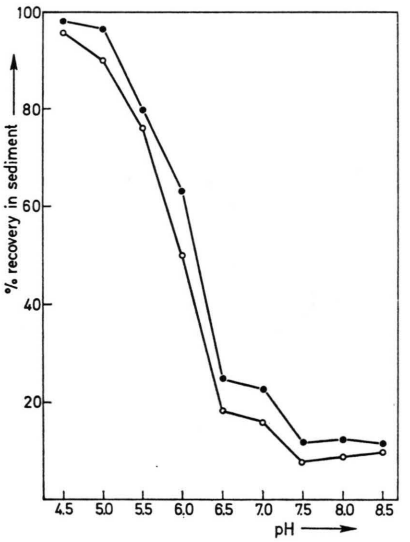


Fig. 1. Sedimentation of a recombined lipoprotein as a function of pH. (●) : Protein, (○) : phospholipid recovered in the pellet. Centrifugation was done for 15 min at $20\,000 \times g$. Solvent: 100 mM NaCl, 10 mM Tris-maleate/NaOH, 0.5 mM EDTA.

bution of protein and phospholipid among pellet and supernatant was determined. A typical result is shown in Fig. 1. Apparently, at pH values around 5, nearly all of the protein and lipid of the samples is sedimented. However, with increasing pH an increasing amount of material is left in the supernatant. At pH >7 the fraction of nonsedimenting material reaches approx. 90% of both protein and lipid. Equilibrium density gradient centrifugation of samples at pH 8.5 revealed that the lipoprotein did not decompose at the higher end of the pH range used: practically all of the material was found in a strongly opalescent band centering around the density of the original pH 5.5-recombinate, and only a very faint band of free lipid could be observed. This latter material most probably was trapped in the flocculated pH 5.5-recombinate used as a starting material.

The results described above show that precipitation of the isolated lipoprotein is limited to certain conditions. Moreover, it is a reversible effect.

ESR studies

Spin labels have been shown to be sensitive markers of the mobility of the labelled groups and thus of molecular structure [9, 10]. They have also been used to investigate the mobility of lipids and protein side chains in recombinates from the components of erythrocyte membranes [11, 12]. In the present paper, we have studied recombinates prepared at pH 5.5, 7.3, and 8.5. In agreement with the results reported earlier, at all pH values lipoproteins of practically equal density were obtained. The state of aggregation of the samples, however, was different: at pH 5.5 the samples were heavily flocculated, whereas, at higher pH, the samples were opalescent but not precipitated [3–5]. When studied in the ESR spectrometer, the spectra of the three samples were found to be different (Fig. 2). At pH 5.5, the spectra were dominated by bands typical of a strongly immobilized label (*e. g.* the low field band of Fig. 2 a) [9–12], whereas at the higher pH values, the bands corresponding to mobile labelled groups became more prominent (Fig. 2 b, c). However, when the samples recombined at pH 8.5 were brought to pH 7.3 or 5.5, the spectra changed to that observed in the pH 7.3- and pH 5.5-recombinates, respectively. Completely analogous results were found when the pH values of the other recombinates were varied.

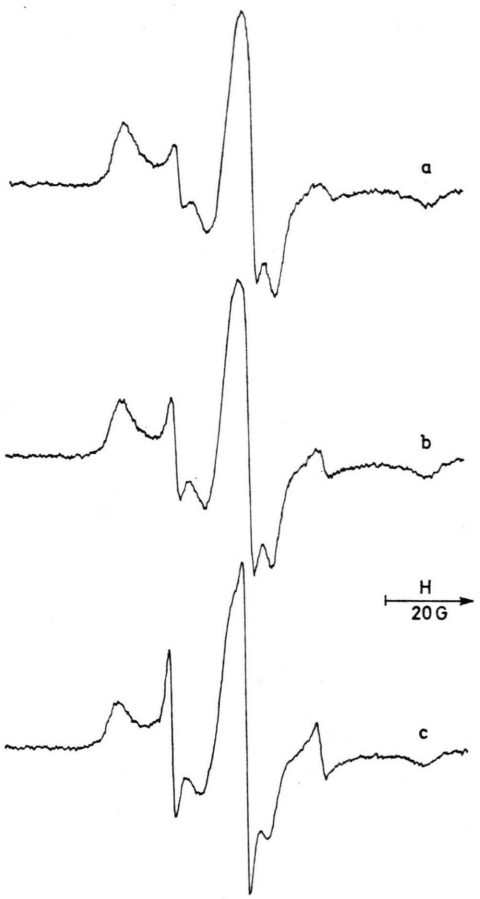


Fig. 2. ESR spectra of spin-labelled protein-lipid recombinates prepared at pH 5.5 (a), 7.3 (b) and 8.5 (c) and measured at the pH of preparation.

In all cases, reversal of pH to the starting value also restored the original spectra. An example of the effects described is given in Fig. 3.

The changes of pH described above were followed by changes in the state of aggregation of the recombinates. Thus, the recombinates prepared at pH 7.3 or 8.5, when brought to pH 5.5, started to precipitate. On the other hand, when samples were brought from pH 5.5 to the higher pH values, they remained in a precipitated form unless they were subjected to a brief sonication. The spectra, however, were not influenced by the transition from the precipitated to the nonprecipitated form.

Spectra identical to those described above were obtained when the lipoproteins, instead being made by dialysis of protein-lipid mixtures against buffers, were formed by direct addition of buffer solu-

tions to the mixtures or by mixing buffer solutions containing proteins and lipids.

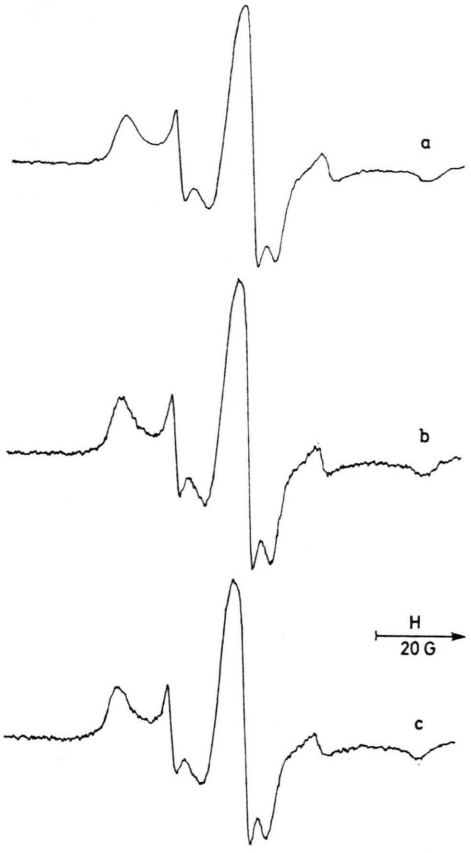


Fig. 3. ESR spectra of spin-labelled recombinates prepared at pH 5.5 (a), 7.3 (b) and 8.5 (c) and measured at pH 7.3. Spectrum (b) is taken from Fig. 2.

Obviously, the molecular structure of the recombined lipoproteins as monitored by the spin label is dependent on pH. This pH dependency is reversible. An influence on the spectra by the state of aggregation of the recombinates could not be detected.

Discussion

The properties of the lipoprotein used in this study depend on pH: 1. At pH values between 4.5 and approx. 6.0, the lipoproteins are easily sedimentable by low speed centrifugation, whereas, at higher pH, the major part of the material does not sediment, in agreement with the results reported earlier [3, 4]. 2. The ESR spectra of a spin label linked to the protein show an increase in its mobility with increasing pH. However, both properties of the recombinates studied depend on pH in a reversible manner. Reversibility is even seen when recombination is performed at different pH values: a recombinate formed at high pH and brought to a low pH shows the same sedimentation behaviour and the same ESR spectra as a recombinate formed at low pH, and *vice versa*.

The results of the sedimentation studies clearly demonstrate that precipitation of recombined lipoproteins is a secondary effect which is not necessarily coupled to the primary effect of lipoprotein formation. The findings of the ESR measurements are at least consistent with this conclusion. Most probably, precipitation is related to isoelectric aggregation of the protein component of the recombinates. This is suggested by the fact that the curve shown in Fig. 1 is similar to the pH profile of isoelectric precipitation of the protein. It is also supported by our ESR measurements where the mobility of the spin label in the recombinates shows the same dependency on pH as reported for the free protein [12]. Especially, the decreased mobility of the labelled groups of the uncombined protein in the isoelectric pH range due to aggregation or to intramolecular conformational changes is paralleled by the behaviour of these groups in the recombined lipoproteins.

Our results show that drawing conclusions about the protein-lipid interactions involved in a recombination process solely from the pH dependence of precipitation may be highly misleading. This comment seems to be especially pertinent with respect to the paper of Barzilay *et al.* mentioned in the introduction [2], since the sedimentation diagrams used by these authors to determine the yield of the recombination process are, in fact, very similar to those of the isolated lipoprotein described here (Fig. 1). It is, therefore, clear that their data do not suffice to characterize adequately their recombination system.

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