Multilayer Formation by a Compressed Monolayer of Poly- ϵ -benzyloxycarbonyl-L-lysine

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Molecular monolayers of poly- ϵ -benzyloxycarbonyl-L-lysine were studied at the air/water interface. Deuterium-exchange measurements and the surface area of the monolayer are consistent with a structure consisting of condensed ordered arrays of α -helices. Collapsed films removed from the surface and air-dried were examined by polarized infrared spectroscopy and electron diffraction and found to consist of molecules in the α -helical conformation. There is no indication of a conformational change during compression of the monolayer, and a series of transitions found in the force-area curve are interpreted as the consecutive formation of additional layers of molecules. Some of the factors that influence this almost perfect plastic behaviour are discussed.

Recent investigations of the surface chemistry of high-molecular-weight synthetic polypeptides have provided good evidence to show that when prepared as monolayers at the air/water interface they frequently exist as condensed ordered arrays of α -helices (Malcolm, 1962, 1965, 1966, 1968*a*). In some instances the force-area curve has a plateau, which has been interpreted as the regular formation of a second layer of molecules (Malcolm, 1966, 1968*a*). The pressure at which this forms is a characteristic of the polymer and depends on the work of adhesion between the polymer and the substrate and on the free energy of the polymer/vapour interface. In a few cases it has been possible to infer the development of a third layer of molecules. It appears that a necessary condition for the formation of a plateau is that the side chains should be sufficiently long and contain groups that interact to promote a co-operative transition. Thus for polypeptides with hydrocarbon side chains only an inflexion is observed in the force-area curve unless the side chain has four or more carbon atoms. Plateaux are observed, however, with the methyl, ethyl and benzyl esters of poly-L-glutamic acid (Malcolm, 1968*a*) and with poly- β -benzyl-L-aspartate (Malcolm, 1968*b*). It therefore seemed probable that poly- ϵ -benzyloxycarbonyl-L-lysine would exhibit a similar pattern of behaviour and a plateau was indeed observed, but in this instance there is remarkable evidence for the regular consecutive formation of additional layers of molecules and it provides a noteworthy example of almost perfect molecular plasticity.

MATERIALS AND METHODS

The material used was obtained from Pilot Chemicals Inc., Watertown, Mass., U.S.A. (lot L-78; mol.wt. 250 000). Solutions for spreading monolayers were prepared by dissolving about 10 mg. of polymer in 1 ml. of dichloroacetic acid and making up to 10 ml. volume with chloroform. In this solvent the polymer is in the α -helical conformation (Karasz, O'Reilly & Bair, 1965). For deuterium-exchange experiments the polymer was deuterated by using *O*-deuterated dichloroacetic acid, and benzene in place of chloroform. Monolayers were prepared with about 0.06 ml. of the solution. These procedures have been described previously (Malcolm, 1968*a*).

Force-area measurements. The type of film balance employed is particularly suitable for this work. It consists essentially of a thin bent metal strip mounted with its lower edge in the interface, forming a flexible barrier across the Langmuir trough (Malcolm & Davies, 1965). When the monolayer is compressed against it, the strip is displaced and exerts a steady reaction on the film. The displacement of the strip, which is measured with a microscope fitted with a micrometer movement, is a measure of the force on the film. The film was compressed continuously with a waxed glass barrier moving at 4 mm./min. and readings were taken at $\frac{1}{4}$ min. intervals. Although the results described below could probably have been obtained with other forms of apparatus, the continuous pressure applied to the film by this method probably gives better results than the usual procedures, where the film is compressed in increments. The force-area curves obtained are essentially dynamic, but the main features of the results are not at all sensitive to the rate of compression.

Observations on collapsed films. It was found that, if a monolayer is compressed until it forms a narrow strip between two barriers, high orientation frequently develops,

the molecules becoming aligned parallel to the barriers. The film can then be removed and examined by electron diffraction (Malcolm, 1968a). This method was applied here and extended to provide thicker oriented specimens for examination by polarized infrared spectroscopy. For this a two-pronged fork, suitably supported at its upper end, is lowered into the water while the film is being compressed. When the barriers are about 3 mm. apart they are gently moved back, releasing the film, and the fork is rotated and moved across the water so that the polymer strip is wound around it, forming a series of loops on the water surface. The barriers can then be used, if necessary, to compress the loops, and the film is picked off the surface by drawing an AgCl plate through the interface between the prongs of the fork. Infrared spectra of dry films so obtained were recorded on a Unicam SP.200 spectrometer fitted with a selenium polarizer and an NaCl prism.

Angle of contact. The angle of contact of the polymer against water was measured with the dipping-plate technique as described previously (Malcolm, 1968a). Films of polymer for this were cast on microscope slides from solution in chloroform containing about 40% (v/v) *NN*-dimethylformamide.

RESULTS

Force-area curve. Monolayers spread on 0.01*N*-hydrochloric acid and on distilled water gave similar force-area curves within the limit of experimental error (Fig. 1). An alternative way of plotting the data is also shown, where the product FA is plotted against A (F in dynes cm.⁻¹, A in Å²/residue). This is essentially an idealized stress-strain curve (Malcolm, 1966), and additionally has the practical advantage of enabling detail to be recognized in the steeply rising part of the force-area curve. Extrapolation of the first steep rise of the curves to zero gives an area of 24.0 ± 0.5 Å²/residue for the monolayer.

Minima occur in the FA - A curve at areas of approx. 12.1, 8.4, 5.9, 4.3 and 3.4(?) Å²/residue. These can be interpreted as corresponding to the completion of two, three, four, five and (possibly) six layers of molecules. When a layer is complete, FA is a direct measure of the stress being applied to the film (at other areas the film is of non-uniform thickness and the stress uneven). The peaks in the force-area curve are accentuated by lowering the temperature of the trough to 16° and almost disappear if measurements are made at 34°. Similarly, lowering of the rate of compression of the film produces smaller peaks. Such variations in the conditions do not, however, significantly alter the positions of the minima in the FA - A curve.

Infrared spectroscopy and deuterium exchange. The spectrum of a collapsed monolayer (Fig. 2a) agreed in detail with that obtained from an oriented specimen prepared by drying a drop of solution of the polymer in chloroform, the film being stroked at the same time with a glass rod to promote alignment of the molecules. The amide I band

Fig. 1. Lower graph: dependence of area (A) on force (F) for a monolayer of poly- ϵ -benzyloxycarbonyl-L-lysine spread on 0.01*N*-HCl (20°). Upper graph: plot of the product of the force (F) and the area (A) as a function of the area (A).

(1655 cm.⁻¹) has the frequency and dichroism normally found for α -helices; the corresponding band for the amide groups in the side chains is at 1700 cm.⁻¹ and has low dichroism. The NH stretching bands (approx. 3300 cm.⁻¹), the amide II band (approx. 1550 cm.⁻¹) and the amide III band (approx. 1250 cm.⁻¹) contain components from both the 'backbone' and side-chain amide groups. This is shown clearly from the spectrum of the deuterated specimen (Fig. 2b). The amide I band of the 'backbone' has moved to 1652 cm.⁻¹ and a dichroic band has appeared at 1450 cm.⁻¹, as is usually observed for deuterated α -helices (Miyazawa, 1962). On the other hand there remain bands at approx. 1700 cm.⁻¹, 3300 cm.⁻¹, 1540 cm.⁻¹ and 1250 cm.⁻¹, all of which now show little dichroism and are attributable to the side-chain amide group, assuming that the deuterium of the side-chain amide groups has completely exchanged. The absence of dichroism from these bands is probably a consequence of a measure of disorder and diversity of side-chain orientations, which is not unexpected and is probably correlated with the poor

Fig. 2. Polarized infrared spectra of air-dried specimens prepared from monolayers (20°): (a) polymer spread on distilled water; (b) deuterated polymer spread on 0.01 N-HCl. —, Electric vector perpendicular to the length of the specimen, i.e. parallel to the direction of compression of the film; ---, electric vector parallel to the specimen.

crystallinity of the electron-diffraction patterns (see below). The total time the polymer was in contact with water was about 15 min., and leaving a monolayer on the surface of 0.01 N-hydrochloric acid (20°) for 19 hr. produced little change in the spectrum. At a substrate pH 7.2 exchange is almost complete after 9 hr. and at pH 10 in a few minutes. Full kinetic measurements were not attempted, but the pattern clearly follows that observed in previous work, except that on alkaline substrates the exchange is rather more rapid than that observed in the esters of poly-L-glutamic acid (Malcolm, 1968a), probably on account of the hydrophilic amide group of the side chain promoting the exchange.

Electron diffraction. The diffraction patterns of

air-dried specimens prepared from monolayers spread on distilled water show poor crystallinity but considerable orientation, with the usual intensity distribution characteristic of the α -helix, as observed in previous work (Malcolm, 1968a). There is a meridional reflexion at $1.50\text{\AA}$, and equatorial reflexions at approx. $14.3\text{\AA}$ and $8.2\text{\AA}$ that can be indexed as the (100) and (110) reflexions of a hexagonal cell. These two reflexions are of rather variable relative intensity from one specimen to another with the (100) usually weaker than the (110). This is somewhat similar to the situation found in poly- γ -methyl L-glutamate, where a high degree of double orientation occurs and the (100) reflexion, normally (in fibre photographs) by far the strongest, is not observed at all in some instances.

The explanation in this case is probably that, as with poly- γ -methyl L-glutamate, the structure develops with the (100) planes mainly parallel to the water surface.

Viewed in the electron microscope the films, estimated to be 200 Å in thickness, appeared remarkably uniform, except where folding had occurred in the last stages of collapse. This observation suggests that, although consecutive layers of molecules appear to form to a thickness of at least 50 Å, as shown by the force-area measurements, it is probable that this process continues further. Otherwise, if at 50 Å the film were to start to fold and buckle, the resulting texture would have been clearly visible in the microscope.

Angle of contact measurements. The angle of contact, θ , determined against distilled water, was $62 \pm 2^\circ$. If we accept, bearing in mind the reservations that have been expressed (Malcolm, 1966, 1968a), the application of Young's equation as a measure of the adhesion of the polymer to the water, the following expression can be used to calculate the free energy of the polymer/vapour interface ($F_{P/V}$):

$$2F_{P/V} = -\gamma_{L/V}(1 + \cos \theta) + W$$

where $\gamma_{L/V}$ is the surface tension of the water and W the work done on the film to convert 1 cm^{-2} of monolayer into a bilayer. W is numerically equal almost exactly (Malcolm, 1966) to the pressure on the film at the plateau (6 dynes cm^{-1}). Taking $72.8 \text{ dynes cm}^{-1}$ for $\gamma_{L/V}$, $2F_{P/V} = 101 \pm 2 \text{ ergs cm}^{-2}$.

DISCUSSION

The results of the polarized infrared spectroscopy and electron diffraction leave no doubt that when the fully collapsed polymer film is removed from the surface and air-dried it is in the α -helical conformation. If the force-area curve arises from the collapse of molecules in the α -helical conformation, the form of the curve can then be accounted for in a relatively simple way. Caution is, however, necessary, since it is in principle possible for some other conformation in the monolayer state to be converted into the α -helical form on compression, giving rise to a plateau in the force-area curve. It is therefore essential to distinguish between observations related directly to the structure of the monolayer and indirect observations on the collapsed film or a dried monolayer.

Loeb & Baier (1968) have used infrared multiple-reflexion spectra to study the conformation of poly- γ -benzyl L-glutamate monolayers. Although this approach is valuable, it is indirect since it involves transferring the monolayer to a germanium or other optical surface. This transfer may cause a conformational change, particularly if the mono-

layer interacts strongly with the water (as with poly- β -benzyl L-aspartate; Malcolm, 1968b) or is hydrogen-bonded to it. If it is possible to observe slow deuterium exchange, as in the present work, information can be obtained about the hydrogen-bonding in the monolayer. Early work (Malcolm, 1962) has, however, been questioned by Loeb & Baier (1967), who point out that deuterium-hydrogen exchange is complicated by the effect of the environment on the exchanging group and that the effect of the nearby surface has not been independently investigated. The kinetic measurements (Malcolm, 1968a) are, however, good support for this approach. In addition in the present work, the evidently complete exchange of the side-chain amide groups under conditions where the 'backbone' peptide groups do not exchange serves as a useful control, and shows that the two groups must be in different environments. If the polymer were a random coil on the surface, with all the groups free to hydrogen-bond to water, it would be expected that they would all rapidly exchange. It can therefore be concluded that, as in previous work, the slow exchange of the 'backbone' rules out conformations in which it hydrogen-bonds to the substrate water.

The interhelix distance in the collapsed film calculated assuming that the equatorial reflexions arise from the (100) and (110) planes of a hexagonal lattice is 16.4 Å. This is in reasonable agreement with the distance (16 Å) calculated from the area/residue measured in the monolayer, assuming the molecules are α -helical. It should be realized of course, particularly with long side chains, that the packing in a monolayer may not be the same as in the solid state, so that exact agreement is not necessarily to be expected. The observed area/residue is too high for the β -conformation to be acceptable, since this leads to an area in a monolayer of about $17 \text{ Å}^2/\text{residue}$ (Malcolm, 1965).

The direct observations from both deuterium-exchange and surface-area measurement are therefore consistent with the presence of the α -helix in the monolayer, or of some closely related conformation. As in other cases (Malcolm, 1968a) the plateau implies a high degree of order in the monolayer, which on this basis arises directly from the side-by-side packing of the rigid helical molecules. On the other hand, if, as has been suggested for poly- γ -benzyl L-glutamate, a conformational change takes place at the plateau (Isemura & Suzuki, 1967) then in the present case some further explanation is necessary to account for the further inflexions in the force-area curves. The explanation given for the plateau in poly- γ -methyl L-glutamate (Malcolm, 1966) can be simply extended, however, by postulating the formation of further layers of molecules. If this is correct the monolayer micelles

of α -helices contain many molecules, since almost every micelle must form six or more layers on compression. A micelle might therefore contain about 100 or more molecules with an overall size of not less than $1500\text{\AA} \times 1500\text{\AA}$ (assuming no end-to-end aggregation of the molecules). The formation of a multilayer, one layer at a time, can then take place by a simple co-operative process in the same way as suggested for the formation of a bilayer (Malcolm, 1968a), except that it is now necessary to postulate the movement upwards of molecules through the entire thickness of the film. Once, however, the pressure to form the first plateau is reached, where there is sufficient energy to detach molecules from the water surface, very little additional energy is necessary to propagate a movement of molecules through the film. An upper limit, bearing in mind that the measurements are dynamic (and the film therefore not in equilibrium), can be calculated from the force-area curve. For the third layer, the work done on the film above that required to remove molecules from the surface of the water is represented by the area in the force-area curve above the level of the first plateau, over the range where the third layer of molecules is forming. This gives approx. 1 erg for the formation of 1cm^2 of triple layer or $0.03\text{kcal.mole}^{-1}$ of residues transferred. Similarly, the peaks in the force-area curve may be related to the energy for initiation of the next layer of molecules (which requires more work than the propagation of a layer once it is initiated). In these regions, however, the film is clearly metastable and the size of the peaks is dependent on the experimental conditions as well as on the number and rate of formation of sites at which the next layer starts to form.

One factor important in determining the behaviour of the polymer is the free energy of the polymer/vapour interface, which is the main source of energy for the transition from monolayer to bilayer (Malcolm, 1968a). The value obtained is rather higher than in other cases where a plateau is observed, and it is of interest to compare this polymer with poly- γ -benzyl L-glutamate. This shows a plateau at about the same pressure and, as might be expected from the more hydrophobic nature of the side chain, has a significantly lower work of adhesion to water ($W_{P/L}$ 94 ergs cm^{-2}). Thus although the amide group in the side chain of poly- ϵ -benzyloxycarbonyl-L-lysine increases the

adhesion of the polymer to water, it correspondingly increases the free energy of the polymer/vapour interface. This relatively higher free energy might be one factor facilitating the formation of additional layers of molecules in the manner observed. There is, however, one important additional factor, since in this instance the side-chain amide groups at the polymer/vapour interface may adsorb water molecules by hydrogen-bonding. These may have to be displaced when a further layer of helices forms, so it cannot be assumed in this case that the free energy of the polymer/vapour interface is directly related to the energy required to separate two layers of polymer molecules. However, the presence of the water molecules could well be very important in promoting the almost perfect plasticity of the system.

In conclusion, it appears that this polymer fits into the pattern established in previous work, and in particular the remarkable formation of the multilayer is good support for the explanation that has been given earlier of the plateau. Although the side chain of this polymer is not one occurring in proteins it is nevertheless of interest as a model for understanding reactions, such as the deuterium-exchange reaction, at interfaces, and the factors that control the tertiary structure of proteins and intermolecular associations.

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