CONFORMATIONAL STUDIES ON THE MEMBRANE PROTEIN OF SARCOTUBULAR VESICLES*

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Among possible mechanisms responsible for active transport through membranes, one could conceive that a conformational change of a membrane protein is a part of the event causing the directed movement of the ion or molecule to be displaced. Hypothetical schemes involving contractile changes in membrane proteins have from time to time been proposed. Furthermore, Changeux, Thiery, Tung, and Kittel[1] have recently presented a theoretical picture according to which the characteristic properties of biological membranes depend on the lattice arrangement of monomers, and the conformational states of these monomers are determined cooperatively by the interactions among themselves and with specific ligands.

This communication describes explorations of such problems by study of the circular dichroism (CD) of suspensions of purified sarcotubular vesicles from rabbit muscle. The choice of material was made for two reasons. Firstly, (e.g., Hasselbach and Makinose,[2] Ebashi and Lipmann[3]) these vesicles constitute the simplest biological system still displaying a transport activity, and they can be obtained in a highly purified form.[4] Secondly, there are reasons to believe (see *Discussion*) that in these preparations a high proportion of the material represents the actively transporting structure.

The results allow certain conclusions regarding the structural protein in these membranes, similar to those recently derived for other membrane preparations by Lenard and Singer,[5] but differing from the work of these authors in showing the presence of an α-helical conformation without complications, and in achieving dissolution of the monomers in aqueous solvents. Efforts to detect transconformations relatable to the transport function led to negative results.

Materials and Methods.—Sarcotubular vesicles: These were prepared from rabbit skeletal muscle by differential and sucrose-gradient ultracentrifugation with the procedure of Seraydarian and Mommaerts.[4] The active fraction was dialyzed overnight against 1 M sucrose at 2°C. Final vesicle concentrations, determined by the biuret method, were about 6 mg protein per ml. These suspensions can be kept at 0°C for several days without deterioration. Their activities, in terms of calcium uptake and ATPase, were tested with the usual procedures.[4]

Optical measurements: Absorption spectra were recorded in the Cary 15 spectrophotometer, using the sucrose diffusate (or other appropriate reference solution) for the baseline. Circular dichroism was recorded in the modified prototype of the Jasco-Durrum instrument; the methodology is described elsewhere.[6] Both measurements were done at room temperature, usually on the same samples in the same cuvet, which consisted of isotropic Suprasil windows with Teflon spacers setting 25- or 50-μ path lengths; this allowed the use of relatively concentrated suspensions, which is advantageous on account of the absorption and CD of the su-

crose medium at short wavelengths. By means of the tandem-cell method,[6] using soluble protein as the standard substance, it was shown that the turbidity of the suspensions does not influence the CD measurement.

To calculate residual values of the extinction or the CD, a mean residual weight of 120 daltons per amino acid residue was arbitrarily assumed.

Stopped-flow device: For the detection of changes after mixing, a stopped-flow device was built in which one syringe contained a vesicle suspension, the other the medium with the components whose effect was at issue.[7] From the turbulent-mixing chamber, the mixture flowed into a cuvet of Suprasil windows spaced usually at 0.5 mm. Since the response of the CD instrument is slow, no excessive mixing speed was aimed at.

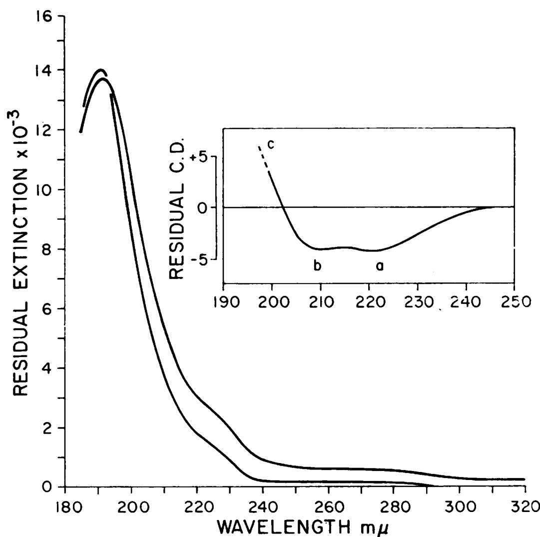

FIG. 1.—*Main figure:* Ultraviolet absorption spectra recorded for a suspension of sarcotubular vesicles (*upper curve*) in 0.5 *M* sucrose, and for the same solubilized by the addition of 1% sodium dodecyl sulfate. Note, in the former case, the finite value of the conservative absorption at longer wavelengths. In the 195-mμ range, the measurements are not accurate due to the light absorption by the additives; it is left unemphasized whether solubilization indeed increases the extinction maximum as the measurements suggest. Ordinate: residual extinction. *Inset:* Circular dichroism; ordinate: residual circular dichroism.

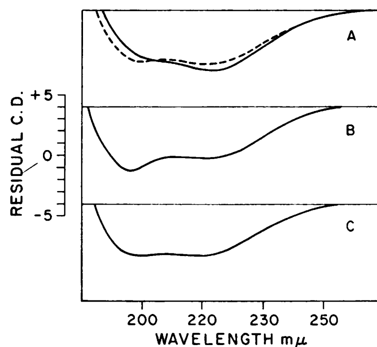

FIG. 2.—Circular dichroism of sarcotubular vesicles, all in 0.5 *M* sucrose. (*A*) Example of a preparation which to a moderate degree shows the modification described in the text. The *a* band is hyperdichroic and the *b* band hypodichroic, and both are bathochromically displaced. The broken curve, for comparison, is that of Fig. 1. (*B*) Same preparation, also containing 1% sodium dodecyl sulfate; the *b* band is increased. (*C*) Same preparation now containing 1% sodium deoxycholate; it is indistinguishable from *A* (broken curve) except for a slight hypsochromicity.

Results.—Ultraviolet spectra: Absorption spectra are given as recorded, and thus, in the case of vesicular suspensions, they contain a contribution due to scattering as can be inferred from the high values above the region of the aromatic absorption bands. Between about 600 and 500 mμ, the scattering is purely "white;" below this range the conservative absorption rises gradually with decreasing wavelength, and thus there is no obvious nonarbitrary basis for correcting the spectra to the consumptive absorption values in the region of interest. Nevertheless, it can be seen (Fig. 1) that the characteristic shoulder near 225 mμ[8, 9] is pronounced, and

that there is no indication of a second shoulder in the 208-mμ region as is found in entirely helical peptides.[10]

When the vesicles are dissolved in detergent (see below), the complication due to scattering becomes much reduced, and the recorded spectra can now be accepted on their face value (Fig. 1), showing the same features as can be inferred for the case of the suspensions themselves.

Circular dichroism: The typical pattern[11] (Fig. 1, *inset*) is one in which the bands *a*, *b*, and *c*[12] are as for the classical case of an α-helix.[11] In the presence of 1 *M* sucrose, whose optically active absorption becomes noticeable, the *c* band is not easily accessible to accurate measurement, but the *a* and *b* bands are of about equal intensity, and are quite typical for the pattern of a predominantly helical protein. The depth of the negative minimum between the *a* and *b* bands varies somewhat in different proteins; as recorded here, it is about as pronounced as usually found, and thus there is no indication of a finite contribution of the β-conformation.[13] These statements refer to vesicles kept in 1 *M* sucrose throughout, and measured in that medium. Occasionally, even preparations in 1 *M* sucrose already show an indication of the modification to be described, as in Figure 2*A*. When the suspensions are diluted into water or salt solution and measured after some standing, or when the vesicles are sedimented centrifugally and resuspended in water or salt solution, the *b* band becomes markedly reduced and merges into a more pronounced positive CD, and both the *a* and *b* maxima show a bathochromic shift of several mμ. These phenomena are somewhat variable, and have not been systematically investigated with respect to their time course and extent; they have not, within the limits of experimentation, gone so far as to lead to the more grossly distorted patterns obtained by Lenard and Singer[5] on erythrocyte membranes. Incidentally, when the vesicles have been stored in sucrose and then sedimented and washed in, for example, phosphate buffer, they are found to give off sizable amounts of protein; this phenomenon has not yet been systematically studied.

Effects of some solvents upon CD: Certain solvents diminish the CD, or may destroy it entirely in the course of time. This has been found at 75 per cent volume concentration for glycerol and ethylene glycol, and, measurable till about 235 mμ, for dimethylsulfoxide.

Various detergents solubilize the vesicles and give rise to entirely clear solutions.[14] This has been studied for sodium dodecyl sulfate and deoxycholate and for the nonionic detergent Triton X-100, each present in a final concentration of 1.0 per cent besides 0.5 *M* sucrose. Absorption spectra have already been given in Figure 1. It is seen (Fig. 2*B*, *C*) that the CD spectra are essentially the same as they are for the vesicular state. Specifically, the CD patterns in deoxycholate and in Triton are approximately identical with those of intact vesicles; that in dodecyl sulfate shows an intensification of the *b* band which, being a feature not otherwise known in CD studies, is hard to interpret. It is known, however, that detergents can alter protein conformation by either suppressing or promoting helical and other structures. Some proteins are not affected; others undergo sizable changes.[15]

CD under conditions of calcium transport: The possible occurrence of conformational changes correlated with the occurrence of calcium transport was investigated by means of the stopped-flow device. This was done with a number of variations with respect to the concentrations of the added materials and the order of their

additions. For example, syringe I contained a suspension of vesicles of about 1 mg protein per ml in 0.1 *M* KCl, 0.005 *M* MgCl, and 0.002 *M* imidazol, pH 7.2, and about 0.15 *M* sucrose carried in by the dilution of the stock suspension; syringe II contained the same medium without vesicles or sucrose, but with 0.00115 *M* ethyleneglycol bis(β-aminoethylether)-N,N'-tetraacetate (EGTA); the solutions were mixed to fill the observation chamber. Syringe I was then refilled with vesicle suspension as before, syringe II with the original medium now also containing 0.01 *M* adenosine triphosphate (ATP) and 0.0010 *M* $CaCl_2$. The apparatus was then placed in the instrument and the basic CD recorded at 224 mμ to provide a reference value. The newly filled syringes were then ejected into the observation chamber, and the CD recorded, now under conditions of continuous calcium transport. There has been an occasional suggestion that ATP alone may cause a slight increase in the CD; this could not be established with certainty, but the addition of calcium did not cause a further alteration (Fig. 3).

Discussion.—Before considering the detailed results presented in this paper, it will be useful to give certain accessory arguments which led to the investigation in this specific form. In general, one would not necessarily expect conformational studies even on highly purified membrane preparations to be informative about the properties of specific functional sites if these comprise only a small fraction of the material. Such estimates as have been made of the surface density of the tetrodotoxin-sensitive sodium channels in nerve[16] or of ionic transport sites[17] or water channels[18] in erythrocytes have indicated low occurrence ratios of these units. Thus, while the recent report by Lenard and Singer[5] on the CD of erythrocyte ghost and bacterial cell membranes is of interest as preceding the present publication, it is not clear to what extent it refers to a functionally active part of those membranes. In the sarcotubular vesicles, by contrast, it may be concluded that the frequency of active sites is high. Studies in this laboratory on the amount of sulfhydryl groups essential for the transport function[19] have suggested that the membrane consists of units of about 120,000 daltons, whereas Martonosi[14] has estimated one calcium-binding site per 300,000 daltons of protein; this leaves little room for inactive protein.

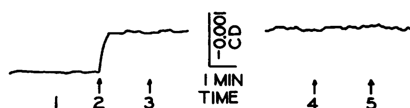

FIG. 3. Example of stopped-flow experiment. Time from left to right, negative CD upward. Path length 0.5 mm. Scales inserted. At *1*, baseline with medium only. At *2*, mixing of vesicles with medium; composition is now 0.4 mg of vesicular protein per ml, in 0.1 *M* KCl, 5 mM $MgCl_2$, 0.02 *M* imidazole buffer pH 7.2, 0.55 mM EDTA; the response at *2* illustrates the transient time of the recording. At *3*, second identical mixing, no change. During the interruption, a mixing was done into the same medium including 5 mM ATP; no change as seen after resumption of the tracing. At *4*, mixing into the same ATP-containing medium, now also containing 0.5 mM $CaCl_2$; at *5*, second identical mixing.

This high surface frequency of transport sites in the sarcotubular vesicles is functionally understandable. In other cell membranes, it seems purposeful to limit the exchange sites to a certain minimum, and in the nerve membrane it would be economical to restrict this to the smallest number of channels required to sustain a self-propagated impulse. The sarcotubular vesicles, on the other hand, appear to have the task of ejecting and reabsorbing massive amounts of calcium in a short time, and thus may be specialized toward a high surface density of its transport mechanism.

At the descriptive level, we first note that the CD spectrum of sarcotubular matter is indeed that of a protein, and does not suggest any superimposed structural feature such as the "form optical activity" displayed by certain cholesteric layer phases[20] or in concentrated solutions of actin.[21] The necessary thickness to provide either a stack of layers with progressive angular offset of an optical axis[22, 23] or a twisted aggregate or tactoid as presumably occurring in actin[21] is not provided by the picture of cell membranes as thin sandwich or mosaic layers[5] of protein and lipid. Our CD spectra, when obtained with well-preserved vesicles, equal the pattern described by Holzwarth and Doty[11] for the right-handed α-helix in synthetic peptides and also found in proteins[11] among which myosin[12] and rhodopsin[24] are of direct physiological interest. On the basis of empirical calibration,[11] one would conclude that about 40 per cent of the protein is in the α-helical conformation. The CD spectrum shows no indication of the one other structural principle, the β-form, that has so far been characterized by this method.[13]

Thus, the vesicular membrane protein gives a far closer approximation to an α-helical pattern than the membranes studied by Lenard and Singer,[5] in which the assignment of this structure involves some extrapolation; upon deterioration in less favorable media, the sarcotubular vesicles change their CD spectrum in the direction of that modified pattern. There is no explicit interpretation for that changed spectrum which has become ascribed to aggregation.[25] Indeed, in myosin this change occurs when the protein becomes demonstrably aggregated,[26] but in the present instance it is not clear just what association would be involved; presumably it implies an arrangement within the membrane.[5]

One major finding is that no modifications were detected that can be related to the calcium-transport activity. It is not easy to remove all calcium from these membranes; a residual quantity is attached. But the presence of added calcium or of EGTA, and of ATP, in different combinations so as to establish steady transport, caused no change. This negative outcome may, of course, require correction in the future; assuming it to be final, it still does not lead to very definite conclusions other than the restatement that under the conditions of the experiment the α-helical parts of the membrane protein showed no sizable alteration. This does not mean for certain that no transconformation occurs as a part of the translocation process. It could be that the relevant structure remains in the same state, both at rest and during the preponderant part of the duty cycle, and is modified only during a small part of the latter; means might eventually be found to influence this in the opposite direction. Also, notwithstanding the argument to the contrary, the active structures may still represent too small a fraction of the material. It could equally be that extensive changes occur, but are restricted to nonhelical regions of the molecule. The possibility also exists, however, that the transport process indeed does not make use of a change in organization of the peptide chain.

Another negative conclusion follows with respect to the theory proposed by Changeux *et al.*,[1] the key assumption of which is that in a membrane made up by the association of globular lipoprotein units "the conformation of these units differs when they are dispersed in solution or organized into a membrane structure." This assumption was based upon the finding[5] that when dissolved in acidic chloroethanol, certain membranes assume an all-helical structure. This is not a valid argument, for the comparison made is not between dissolved and nondissolved, but between

presence in chloroethanol and in a milieu which is not helix-forming. A valid comparison seems to be offered by dissolution in detergents as described here. The resulting solution is heterogeneous[27] and evidently the vesicles contain more than one kind of protein, but there is no doubt that they are molecularly dispersed and that no structured material remains. These solutions have the same CD as the original vesicles; only small secondary deviations occur such as may easily be induced by detergents.[15] To be sure, the interpretation of this finding is subject to the same limitations as were given above for the absence of a demonstrable change in relation to ion transport. The result, therefore, does not exclude conformational changes, other than the method detects, between the protein in the membrane and in solution, and thus it does not occasion a denial of the theory under consideration; but it does remove the one major experimental basis upon which it is founded.

Summary.—Circular dichroism measurements show that the dominant structural feature of the protein of sarcotubular membrane is the α-helix, which constitutes about 40 per cent of the protein matrix. This undergoes no demonstrable change during calcium transport, and shows no difference whether the membrane is intact or is dispersed in detergent.

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