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BACTERIORHODOPSIN, BOUNDARY LIPID AND PROTEIN CONFORMERS

A SPIN LABEL STUDY

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Summary

A spin label study, as a function of temperature, has been made with the bacteriorhodopsin membrane using a stearic acid spin label. The ESR spectra show a strong variation with temperature and the presence of isosbestic points. The spectra are interpreted as indicating the presence of a two-component system with an activation energy (approx. 14 kcal/mol) corresponding to a protein conformational change. This activation energy is similar to that deduced from recent flash photolysis studies.

It is concluded that the spin label is sensitive to the temperature-dependent protein conformational change in this membrane system.

Spin labels, despite difficulties associated with their inherent perturbation characteristics have been shown to be useful as probes to gain insight into the structure and dynamics of lipids and proteins in membranes [1, 2]. Spin-labelled fatty acids or lipids have been used to study the dynamics of the lipid system within natural and reconstituted membranes [3, 4]. Intrin-

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sic protein—lipid systems, when studied above the main transition temperature $(T)_c$ of the respective lipid or lipid mixture, give rise to an immobile ESR component, which was assumed to indicate lipid molecules adjacent to these proteins [4—9]. In order to study in more detail the physical state of lipids adjacent to an integral protein, rhodopsin, Davoust et al. [10] bound a spin label covalently to this protein. In all these studies it is implicitly assumed that a change in the protein conformation caused, for example, by temperature, pressure, etc., may not be reflected in the dynamics of the spin label.

We will show in this paper that the molecular motion of a spin label attached to a fatty acid chain and incorporated into a biological membrane, is influenced by a protein conformational change. Temperature-dependent changes in intrinsic proteins have been found for various proteins such as rhodopsin [11, 12], bacteriorhodopsin [13, 14] and the Ca^{2+}-ATPase from sarcoplasmic reticulum [15—18].

We chose for our studies the purple membrane from *Halobacterium halobium*, one of the best-characterized natural membrane systems. Only a single protein, bacteriorhodopsin, which spans the bilayer of the membrane, is present. It acts as a light-driven H^+ pump as a result of a cyclic photoreaction [19]. The proteins are arranged in a two-dimensional lattice structure, P_3 [20], and are immobile at their lattice points [21, 22]. The lipid:protein molar ratio is particularly low. Only 12—14 lipids per protein are found [23] with no preferred affinity of any lipid class to the protein [24].

We have recently provided evidence based upon our flash photolysis studies that conformational transitions occur in bacteriorhodopsin [13]. The double-exponential decay behaviour for one of the transients, M_{412}, as well as the temperature-dependence for transient, $'O'_{640}$, was explained by the hypothesis that bacteriorhodopsin exists in a temperature-dependent equilibrium between two conformational forms. The two forms are then considered to determine two different reaction pathways. The idea of two different bacteriorhodopsin conformers has support from another laboratory [14].

The growth of *H. halobium* R1 (kindly supplied by Dr. R. Henderson) and purple membrane preparation was according to the procedure described by Oesterhelt and Stoeckenius [25]. The collected purple membranes from the density gradient were washed in 150mM KCl/20mM acetate buffer, pH 5, and used in this condition. For ESR measurements the purple membranes were sedimented and incubated at room temperature with an *N*-oxyl-4′,4′-dimethyloxazolidine derivative of 16-ketostearic acid as spin label. The samples contained between 1 and 2 mol spin label per 100 mol lipid.

ESR spectra were recorded on a Varian E9, 9 GHz spectrometer, equipped with an N_2 gas-flow temperature-regulation system. The spectra were digitised (1000 points) and stored on cassette using a Varian 620/L 100 minicomputer with 20K core memory. The same system was used for integrations and spectral subtractions. The experimental spectra were shifted both horizontally and vertically for a common centre-point.

The ESR spectra for our stearic acid spin label incorporated into the purple membrane are shown as a function of temperature in Fig. 1. It can be seen from the spectra that a marked temperature-dependence of the line-

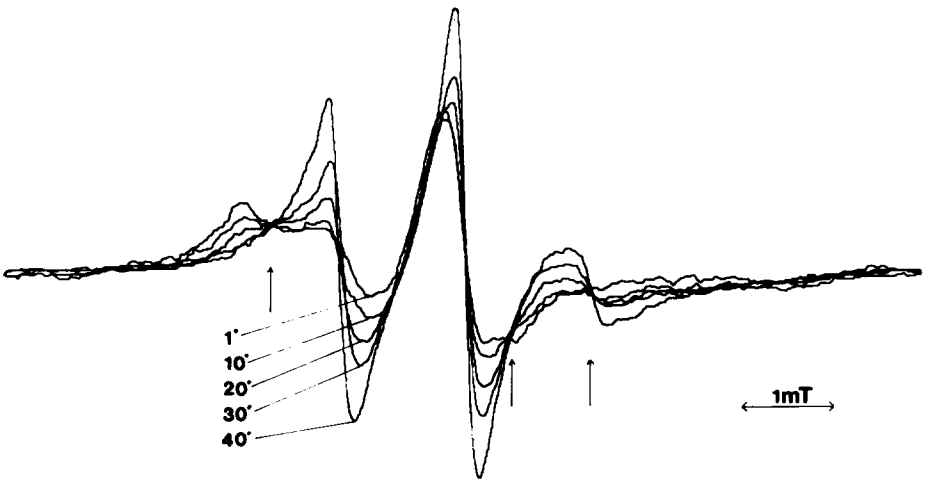

Fig. 1. ESR spectra of a stearic acid spin label [1, 14] incorporated into purple membranes from *H. halobium* at different temperatures.

shape occurs. At low temperatures an immobilised component is indicated which decreases as the temperature is raised. Although the double integrals of the experimental spectra were all within 12% of the mean, the double integrals were normalised for the spectra shown. The experimental findings are in agreement with the results obtained by Chignell and Chignell [26].

One characteristic of the set of spectra in Fig. 1 is the occurrence of at least three non-baseline isoclinic (or isoslopic) points as arrowed. From this result we deduce that the same number of isosbestic points in the respective ESR spectra is occurring [27], which indicates that the measured ESR spectra are the composite of two different spectra. It should be noted that no isoclinic point would be obtained if one or both spectra showed a significant temperature-dependence of their line-shape.

It is possible to determine the proportions of fluid and immobile components from a measured composite spectrum, either by subtraction or by combination. In the subtraction method the spectrum of one component is subtracted from the composite spectrum until the spectrum of the second component is obtained. Regardless of which method is used, the a priori assumptions in evaluating the composite spectra are: knowledge of at least one component (subtraction method) or both (combination method) and that no change with temperature occurs in line-shape of the individual component spectrum. The almost perfect isoclinic points rule out any marked temperature-dependence in the line-shape of either of the spectra, so limitations mentioned by Jost and Griffith [28] against the 'fluid component' subtraction method are therefore not relevant to our system. However, we do not know whether at the highest and lowest temperature (40 and 1°C, respectively) we are obtaining a single component spectrum: (heating to even higher temperatures or the use of glycerol in order to measure at lower temperatures is not possible, as in both cases changes in the purple membrane are occurring (Hoffman, W., unpublished observations)).

We therefore evaluate our spectra using the subtraction method in the

following way. (a) The spectrum obtained at 40° C was subtracted in various proportions from the spectra measured at lower temperatures. The endpoint of subtraction for this method is reached by the occurrence of phase reversal of the narrow fluid component line. As the spectrum at 40° C may contain some immobile component this method provides an underestimation of the amount of immobile component. (b) We also subtracted the spectrum measured at 1° C from the spectra at higher temperatures. This evaluation provides an underestimation of the mobile component. Using the first method we obtain 5% of fluid component at 1° C and from the second method we obtain 20% of immobile spectrum at 40° C. (These are lower limits in each case.) These values are used to calculate the fraction of immobile and fluid components at various temperatures, and are given in Table I.

TABLE I

Computer analysis of the ESR spectra measured at various temperatures by the subtraction methods a and b as described in the text. The error to obtain the endpoint is 10% (*, method b) and 5% (, method a).**

T (°C)	Method a		Method b	
	% (40° C spectrum)** of total	% fluid = % (40° C) × 0.8	% (1° C spectrum)* of total	% immobile = % (1° C) × 0.95
1	5	4	—	—
10	15	12	70	66.5
20	25	20	60	57
25	37.5	30	45	42.75
30	50	40	35	33.25
40	—	—	20	19

The temperature-dependence for the calculated ratio of fluid:immobile component is shown in Fig. 2 in the form of a van't Hoff plot for both methods a and b. The reaction enthalpies are 15.1 and 13 kcal/mol (1 kcal = 4.18 kJ), respectively. The true temperature-dependence should lie between the two lines seen in Fig. 2 which provide only upper and lower limits.

Also shown in Fig. 2 is the temperature-dependence for the equilibrium constant proposed for two bacteriorhodopsin conformers based upon our flash photolysis studies [13]. The reaction enthalpy in this case is 16.7 ± 2.3 kcal/mol when calculated from the temperature-dependence of transient $'O'_{640}$. A slightly lower value of 13.7 ± 2.5 kcal/mol is obtained from the temperature-dependence of two different M_{412} forms. The reaction enthalpies obtained by flash photolysis and ESR methods are similar (within experimental error).

Chignell and Chignell [26] suggested that the highly immobile component corresponds to boundary lipid. If this were the case one might expect that a continuous change in spin-label motion would occur with temperature rather than the observed occurrence of a two-component change with isosbestic points (see Figs. 1 and 2). We do not know where the spin-labelled molecule is situated because of the very high protein to lipid content of the biomembrane structure. Nevertheless, the conclusion that a protein conformation change occurs explains the presence of these isosbestic points and corresponds to an appropriate activation energy for the process.

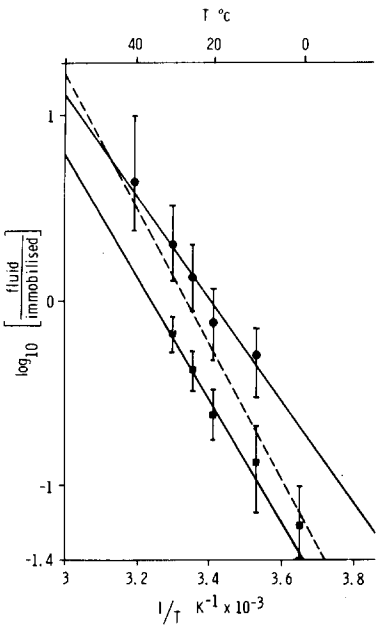

Fig. 2. Van't Hoff plot for the ratio, fluid:immobile ESR component, calculated according to method a (■——■) and method b (●——●) as described in the text. Straight lines are obtained by linear regression yielding reaction enthalpies of 15.1 kcal/mol (■——■) and 13.0 kcal/mol (●——●). Interrupted line is the temperature-dependence of the ratio between two bacteriorhodopsin conformers and is redrawn from Ref. 13.

The occurrence of fluid and immobile ESR components observed in biomembranes and reconstituted systems is usually attributed to the presence of the spin label within fluid and boundary lipid regions with the protein assumed to provide a boundary wall [4—9]. Our studies show that the changes in conformation of the protein may well provide a dominant role in determining the occurrence of the two ESR components, at least in this membrane system.

Rhodopsin from the disc membrane of vertebrate rod outer segments displays a similar type of conformational change with reaction enthalpies between 10 and 30 kcal/mol depending on solution conditions [11, 12]. This conformational change occurs as well when rhodopsin is detergent-solubilized and contains less than 0.2 mol phospholipid per mol rhodopsin [29]. Thus, this type of conformational change is a feature of the protein itself and not, particularly, induced by a lipid phase change. In a recent study by Davoust et al. [10] a spin-labelled fatty acid was covalently bound to reconstituted rhodopsin in egg lecithin vesicles. These workers show a change with temperature and with variation of the protein to lipid ratio of the amount of observed immobile ESR component. A protein conformeric change of rhodopsin as a function of temperature may also need to be taken into account in this system.

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