Immobilization of a Spin-Labeled Fatty Acid Chain Covalently Attached to Ca^{2+}-ATPase from Sarcoplasmic Reticulum Suggests an Oligomeric Structure†

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ABSTRACT: A spin-labeled fatty acid chain (16-doxylstearic acid), linked by an ester bond to a maleimide residue, was covalently attached to the Ca^{2+}-ATPase of rabbit sarcoplasmic reticulum. Three different hydrophobic environments of Ca^{2+}-ATPase were investigated by electron spin resonance: (i) native sarcoplasmic reticulum, (ii) reconstituted vesicles obtained by exchange of the endogenous lipids with egg lecithin, and (iii) Ca^{2+}-ATPase solubilized by the nonionic detergent octaethylene glycol monododecyl ether ($C_{12}E_8$). All spectra were composite, with a major component corresponding to strongly immobilized probe (called "immobilized component"). The percentage of immobilized component seemed to decrease with increasing temperature or in the presence of a small amount of $C_{12}E_8$, under nonsolubilizing conditions. Quantitation of the percentage of immobilized component was performed by linear combination of pairs of appropriately selected spectra, for which it could be assumed that changes predominantly were caused by differences in the proportion of immobile and mobile components and not by changes in line shape. This was the case either by a small variation in temperature or by addition of small amounts of $C_{12}E_8$. The immobilized component generated by the combination of such spectra is characterized by a large splitting with only a moderate temperature dependence (from 66 G around 4 °C to 63 G around 35 °C). Partial immobilization persisted after solubilization of ATPase with $C_{12}E_8$. Ultracentrifugation studies demonstrated that the immobilized component was associated with the presence of oligomeric ATPase in the solution. Saturation transfer electron spin resonance revealed a rotational correlation time of 5×10^{-5} s (at 15 °C) for the immobilized component in complexes with egg phosphatidylcholine. This value is close to the rotational correlation time of the protein as determined by saturation transfer using a short-chain maleimide spin-label. We propose that the immobilized component arises from lipid chains trapped in protein oligomers, which exist in native sarcoplasmic reticulum. Partial dissociation of the oligomers may be induced by a rise of temperature or by perturbation of membrane structure with a low concentration of detergent.

The aggregational state of membrane proteins is currently a topic of great interest, especially in the case of transport proteins where self-association may be of great importance for the transport function (Martonosi et al., 1977; Singer, 1974; Kyte, 1975). Information on self-association is also an important factor in all discussions concerning lipid–protein interactions in membranes. Lipid chains trapped between a functionally organized group of proteins are likely to have a different degree of mobility than lipid chains surrounding a monomeric peptide (Chapman et al., 1979; Davoust et al., 1980). Nevertheless, protein oligomerization has generally not been taken into account in recent discussions of the physical state of lipid in the vicinity of membrane proteins such as sarcoplasmic reticulum Ca^{2+}-ATPase[1] (Hesketh et al., 1976; Rice et al., 1979). This is a pertinent question, since various lines of evidence now suggest that this protein occurs as an oligomer in the membrane: detergent solubilization (le Maire et al., 1978; Møller et al., 1980), freeze–fracture (Scales & Inesi 1976,a,b; Jilka et al., 1975), fluorescence energy transfer (Vanderkooi et al., 1977), and flash photolysis studies (Hoffmann et al., 1979; Cherry, 1979; Bürkli & Cherry, 1981). Also, in a recent ESR study of sarcoplasmic reticulum membranes the possibility of Ca^{2+}-ATPase aggregates was considered by Nakamura & Martonosi (1980).

In this paper we will show that a long-chain spin-labeled fatty acid covalently attached to Ca^{2+}-ATPase can be used to differentiate between monomers and large oligomers of the protein. A spin-labeled alkyl chain can be covalently bound to an intrinsic protein if the alkyl chain is coupled to a reacting group such as a maleimide (Favre et al., 1979; Davoust et al., 1979), an isocyanate (Davoust et al., 1980), or a nitrene (Fellmann et al., 1980). In previous work we have used such spin-labels with a nitroxide near the ω-2 terminal carbon and a reactive group on the polar moiety. The ESR spectra after binding to rhodopsin, in disc membranes or in reconstituted systems, showed essentially narrow lines at 37 °C, indicative of a fast motion ($\tau_c \simeq 10^{-9}$ s) (Favre et al., 1979; Davoust et al., 1979). Similarly, using the same approach, Swanson et al. (1980) have obtained narrow lines with hydrophobic long-chain spin-labels covalently attached to cytochrome oxidase in reconstituted vesicles. However, under conditions where protein aggregation took place, an immobilized component with a large splitting ($\simeq$65 G) became visible. In the case of rhodopsin, protein aggregation seems to be a nonphysiological phenomenon which can be produced by one of the three following processes: (a) a decrease of the lipid to

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[1] Abbreviations used: Ca^{2+}-ATPase, Ca^{2+}-activated, Mg^{2+}-dependent adenosinetriphosphatase; $C_{12}E_8$, octaethylene glycol monododecyl ether; ESR, electron spin resonance; Tris, tris(hydroxymethyl)aminomethane; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; DEAE, diethylaminoethyl; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; (1,14)MSL, long-chain spin-labeled maleimide (see formula in text).

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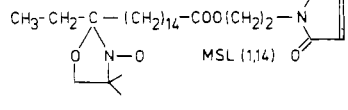
protein ratio; (b) prolonged illumination at high temperature; (c) a decrease of temperature (Favre et al., 1979; Davoust et al., 1979, 1980). In the case of cytochrome oxidase, it is not clear whether the physiological state (in mitochondria) corresponds to protein oligomers. In the reconstituted systems, it depends very much on the method of reconstitution (Swanson et al., 1980).

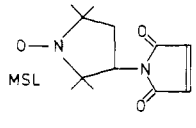
Recently, we have attached a fatty acid spin-label to Ca^{2+}-ATPase in purified sarcoplasmic reticulum membranes by taking advantage of photoaffinity spin-labeling (Fellmann et al., 1980). We found a very large immobilized component in the ESR spectra at 37 °C, corresponding to ~60% of the signal. Thus by means of this spin-labeling technique Ca^{2+}-ATPase differs greatly from rhodopsin. In the present study a spin-labeled fatty acid was linked by an ester bond to a maleimide and then attached to Ca^{2+}-ATPase. ESR spectra of the labeled protein were recorded under various conditions: in native sarcoplasmic reticulum, in reconstituted membranes, and in detergent. The advantage of the reconstituted system is that it permits complete removal of all unbound spin-labels and contaminating proteins during the process of lipid exchange. The results are therefore easier to interpret. The advantage of the solubilized form of Ca^{2+}-ATPase is that direct information on the degree of oligomerization can be obtained by analytical ultracentrifugation.

Materials and Methods

Spin-Labels. A long-chain fatty acid spin-label (16-doxylstearic acid) prepared by the procedure of Hubbell & McConnell (1971) was linked by an ester bond to a maleimide residue by a modification of the previously described procedure (Favre et al., 1979). In the new synthesis, *N*-(2-hydroxyethyl)maleimide was obtained by cyclization of the corresponding maleamic acid in the presence of 1-hydroxybenzotriazole and *N,N*-dicyclohexylcarbodiimide according to Trommer & Hendrick (1973). The crude product was purified by column chromatography on silica with a 30:5 chloroform–acetone mixture as eluant. The rest of the synthesis was carried out according to Favre et al. (1979). The resulting label [(1,14)MSL] is capable of forming covalent bonds with



sulfhydryl and amino groups of proteins. The short-chain maleimide spin-label (MSL), used especially for saturation



transfer ESR, was purchased from Syva (Palo Alto, CA). Other labels used included the noncovalently binding 16- and 5-doxylstearic acid methyl esters.

Membrane Preparations. Native sarcoplasmic reticulum was prepared by zonal centrifugation from rabbit skeletal muscle as previously described (le Maire et al., 1976). For experiments with reconstituted membranes and solubilized ATPase the native membrane was purified by extraction with a low concentration of deoxycholate by method 2 of Meissner et al. (1973). This preparation gave a single band on gel electrophoresis (Andersen & Møller, 1977). After spin-labeling of the purified vesicles (see below), lipid was exchanged twice in cholate according to the procedure of Warren et al. (1974), with a 5-fold molar excess of egg lecithin (99% purity, Lipoid KG, Papenburg) relative to the endogenous lipids present in the starting material. The efficiency of the method in removing non-protein-bound spin-label was checked with the fatty ester analogue (16-doxylstearic ester) of the long-chain maleimide label. When the ATPase was labeled with this probe, no ESR signal could be detected in the sample after lipid exchange. The ATPase activity in the exchanged preparations was high (around 6 μmol mg^{-1} min^{-1} as compared to 5.5 μmol mg^{-1} min^{-1} for the purified ATPase at pH 7.5 and 20 °C), but no Ca^{2+} transport could be detected. Transport capacity was partially restored after the following reconstitution procedure. Exchanged ATPase (2 mg of protein containing ~0.5 mg of lipid) was added to 2–5 mg of egg lecithin dissolved in 1% cholate in 1 mL of the same buffer as that used for exchange (50 mM Tris, pH 8.0, 1 M KCl, 0.3 M sucrose, and 1 mM NaN_3). After centrifugation for 30 min at 100000*g* the clear supernatant was dialyzed for ~60 h at 5 °C in 1000 volumes of (0.01 M Tes, pH 7.5, 0.4 M KCl, 0.3 M sucrose, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM NaN_3) with frequent buffer change. After dialysis the sample was washed twice in the same buffer, and the concentrated membranes were used immediately for ESR experiments and Ca^{2+}-transport assays. The capacity of the membrane for Ca^{2+} accumulation was 0.3–0.5 μmol mg^{-1} min^{-1} in presence of oxalate and 20 nmol mg^{-1} min^{-1} in absence of any Ca^{2+}-trapping agent. Examination of preliminary freeze–fracture replicas by electron microscopy indicated that the protein was inserted into the lipid vesicles, with a particle density somewhat less than that of native sarcoplasmic reticulum.

Detergent-Solubilized ATPase. Spin-labeled ATPase solubilized by the nonionic detergent octaethylene glycol monododecyl ether ($C_{12}E_8$, Nikko Chemicals, Tokyo, Japan) was prepared in two different ways. One was simply to add $C_{12}E_8$ from a concentrated stock solution (40 mg/mL) to the reconstituted membranes described above until solubilization occurred. This solubilization event was monitored by measuring the protein content of the supernatant after centrifugation for 45 min at 100000*g*. In the second method solubilized lipid was removed by DEAE-cellulose chromatography (Møller et al., 1980). The absence of mixed micelles of phospholipid and detergent in the sample facilitated the analysis of the scans obtained in the sedimentation velocity experiments (see below). Approximately 10 mg of spin-labeled ATPase was dissolved in 20 mg of $C_{12}E_8$ in 2 mL of buffer A (0.01 M Tes, pH 7.5, 0.1 mM $CaCl_2$, and 50 mM NaCl) and applied to a 0.9 × 3.8 cm DEAE-cellulose column (DE-52, Whatman Ltd.) equilibrated with washing buffer (2 mg/mL $C_{12}E_8$ dissolved in buffer A). After 75–100 mL of washing buffer had passed slowly through the column, the delipidated protein was eluted with washing buffer to which had been added 400 mM NaCl. The peak fraction was concentrated by filtration on an Amicon M3 device to a final protein concentration of ~10 mg/mL for ESR measurements. ATPase activity decayed rapidly after elution in the absence of glycerol but could be conserved for several hours when 20% glycerol was added to the buffers (Dean & Tanford, 1978). The presence of glycerol had no effect on the ESR spectra. To ensure that the column method was effective in removing non-protein-bound spin-label, we performed an experiment with the fatty ester analogue of the long-chain maleimide label. No ESR signal was detectable in the eluted protein fractions after concentration. Also, with the maleimide it was routinely checked that the fractions eluted immediately before the protein did not contain any label. For sedimentation of oligomers of ATPase, the concentrated and solubilized sample

was centrifuged for 40 min at 130000*g* on a Beckman airfuge (70 min if 20% glycerol had been added; see above). Aliquots for analytical ultracentrifugation and ESR measurements were taken before and after the centrifugation.

Binding of Spin-Label to ATPase. Native sarcoplasmic reticulum membranes suspended in 1 mM Hepes buffer (pH 7.5) and 0.3 M sucrose at a protein concentration of 12–18 mg/mL were incubated overnight at 5 °C with 0.1 mol of (1,14)MSL/150000*g* protein. The label was added in a small volume (<1% of the total) of ethanol. After incubation the protein was washed twice with 2% BSA in the same buffer and finally with buffer not containing BSA. Lipid extraction according to the procedure of Bligh & Dyer (1959) allowed us to estimate the amount of non-protein-bound label by double integration of the ESR spectra obtained on the extract. We found that the lipid-extractable spin-label ranged between 5% and 20% of the total signal in the sample. Thin-layer chromatography performed as previously described (Favre et al., 1979) indicated that most of the label in the extract was free, i.e., not bound to phosphatidylethanolamine.

For studies of reconstituted and detergent-solubilized ATPase the purified protein was incubated under the same conditions as described above except that 1.5 mol of (1,14)MSL (or in some cases MSL) was used per mol of ATPase (M_r 115 000; le Maire et al., 1976). Double integration of the spectra obtained with the reconstituted system indicated that ~1 mol of (1,14)MSL was bound per mol of protein. This did not appear to affect ATPase activity (see above). The lipid extract of the reconstituted system contained <1% of the total signal. Labeling assay with noncovalently binding fatty ester spin-labels (1 mol/mol of ATPase) was achieved in the same way as with (1,14)MSL either before or after (for ascorbate reduction experiments) lipid exchange and reconstitution.

ESR Experiments. ESR spectra of the samples in 50-μL flat quartz cells were recorded on a Varian E109 spectrometer with a field frequency lock and a temperature control accessory. The spectrometer was connected to a Tektronix 4051 computer for accumulation, storage, and evaluation of the spectra. A few experiments were also carried out on a Varian E3 spectrometer. To estimate the fraction of immobilized label, we performed linear combinations on pairs of appropriately selected spectra and subtracted the resulting "average immobilized component" from the original spectra to determine the percentage of immobile component. In the selection of spectra to be combined, we aimed to compare spectra where the line shapes were constant, and only the proportion of immobilized and relatively mobile label differed. The fulfilment of these criteria is considered in connection with the presentation of the results. Saturation transfer spectra (second harmonic, 90° out of phase) were recorded as previously described (Baroin et al., 1977).

Other Methods. Protein and phospholipid concentrations were determined according to Lowry et al. (1951) and Bartlett (1959). ATP hydrolysis was measured spectrophotometrically at 20 °C by a NADH-coupled assay, using phosphoenolpyruvate to regenerate ATP (Møller et al., 1980). Ca^{2+} transport was measured with $^{45}Ca^{2+}$ in the same assay medium as used for the enzymatic assay (0.01 M Tes, pH 7.5, 0.1 M KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM MgATP, 1 mM phosphoenolpyruvate, and 0.07 mg/mL pyruvatekinase), except that in some experiments 5 mM dipotassium oxalate was added. Five minutes after the start of the experiment, the sample (containing 0.2–0.6 mg of protein/mL) was filtered through a 0.22-μm Millipore filter (Meissner et al., 1973), and the radioactivity of the filtrate was measured by liquid scintillation counting. The Ca^{2+} concentration in the filtrate was calculated by comparison with controls not containing ATP or protein. In some of the reconstitution experiments efficient removal of cholate (<1 mol/mol of ATPase) from the sample was checked by the use of [^{14}C]cholate (Radiochemical Centre, Amersham, England). Ascorbate assays of spin-probe localization were performed at a 5 mM concentration of the reducing agent at 5 °C. Reduction of spin-label was started by addition of 0.25 volume of freshly prepared 25 mM sodium ascorbate, dissolved in 1 mM EDTA and 0.01 M Tes (pH 7.5), which had been bubbled with N_2 until the start of the experiment. Sedimentation velocity experiments were performed in a Beckman Model E analytical ultracentrifuge, equipped with a photoelectric scanner. The samples were scanned in an AN-D rotor during run-up to 44 000 rpm, and sedimentation coefficients were calculated from the movement of boundaries at that speed.

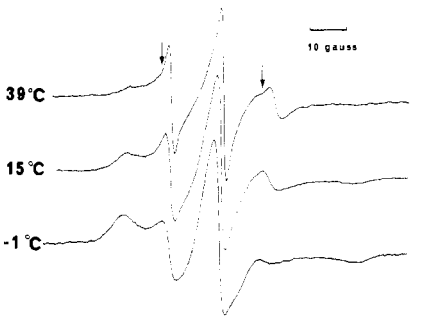


FIGURE 1: ESR spectra of (1,14)MSL spin-label in sarcoplasmic reticulum at various temperatures. The arrows on the top spectrum show the discontinuity revealing the heterogenous character of the narrow component.

Results

Sarcoplasmic Reticulum. Figure 1 shows the ESR spectra of (1,14)MSL in sarcoplasmic reticulum. The spectra are composite, the broad component ("immobilized component") representing a large fraction of the signal even at 39 °C. It corresponds to a splitting of 65 ± 0.5 G at −1 °C and 63 ± 1.5 G at 39 °C. The narrow component ("mobile component") very likely contains at least two subcomponents which are easily recognized at high temperature (arrows on the top spectrum of Figure 1 point out the discontinuity indicative of the dual composition of the lines). The most narrow component can be accounted for by freely diffusing spin-label. For demonstration of this, computer subtraction of a spectrum corresponding to the noncovalently bound 16-doxylstearic ester spin-label incorporated into sarcoplasmic reticulum membranes was carried out until evidence of a phase inversion appeared on the signal. At this stage the spectrum could never be considered to be solely due to the strongly immobilized component. Double integration showed that a constant fraction corresponding to a maximum of 15% of the signal could be subtracted at all temperatures by the procedure described before. This gives an *upper* limit for the percentage of non-protein-bound (1,14)MSL.

Lipid extraction in organic solvent gave a result compatible with the spectral determination, but with a larger uncertainty (5–20%). The results are very similar to the results obtained recently in the same system with a spin-labeled fatty acid possessing a photoreactive group (Fellmann et al., 1980), except that with this spin-label the percentage of unbound label was larger.

In conclusion, a fatty acid spin-label, when covalently bound to sarcoplasmic reticulum membranes, gives rise to two com-

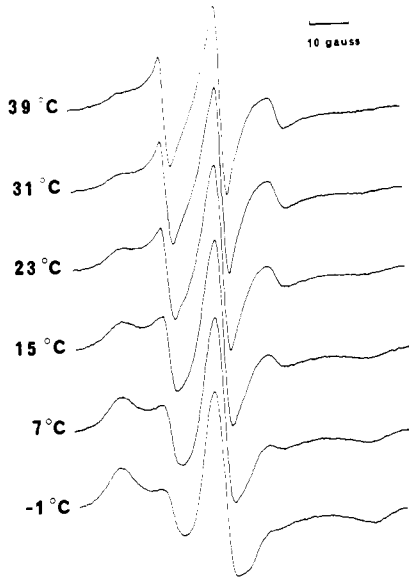


FIGURE 2: ESR spectra of (1,14)MSL spin-label in Ca^{2+}-ATPase–egg phosphatidylcholine complexes at a lipid to protein ratio of 250:1 (mole to mole).

ponents of very different mobility. Therefore, the protein has two different hydrophobic environments, even at high temperatures. A more quantitative description is given in the next paragraph dealing with the reconstituted system.

Reconstituted System: Degree of Immobilization. The ESR spectra of (1,14)MSL bound to Ca^{2+}-ATPase, exchanged and reconstituted with egg lecithin (mole ratio of protein to lipid 1:250), are given in Figure 2. Qualitatively, the appearance of the spectra is very similar to that obtained at the same temperature in the native sarcoplasmic reticulum, except that the narrow component is slightly reduced. This confirms that part of the narrow component in the former system was due to unbound label which has been removed during preparation of the reconstituted system, where the endogenous lipids have been exchanged.

The splitting of the immobilized component again varies only slightly with temperature: 66 ± 0.5 G at −1 °C and of the order of 63 G at 39 °C. Such large splittings are indicative of very long correlation times, which can be measured by saturation transfer ESR (ST-ESR) ($\tau_c > 10^{-7}$ s). The ST-ESR spectra were recorded in reconstituted systems with a covalently bound short-chain spin-label (MSL) and with the covalently bound fatty acid spin-label [(1,14)MSL]. The spectra obtained are shown in Figure 3. The contribution of the weakly immobilized component to the ST-ESR spectrum of (1,14)MSL is not negligible, particularly in the central part. However, the overlap is not substantial in the low-field and high-field regions. It appears that the former spin-label (MSL) is more immobilized than the latter [(1,14)MSL]. But the remarkable fact is that both spectra correspond to rotational correlation times in the range of 10^{-4} s. This numerical evaluation is based on measurements of the parameters H''/H and L''/L according to Thomas et al. (1976). For MSL, which supposedly reports on the protein rotational correlation time (Thomas & Hidalgo, 1978), τ_c was found to vary from $\sim 5 \times 10^{-4}$ s at 5 °C to $\sim 5 \times 10^{-5}$ 10^{-5} s at 30 °C. In the case of (1,14)MSL it was difficult to obtain reliable numbers over this whole range of temperature. Nevertheless, the overall line shapes, with large contributions from the low- and high-field regions, are indicative of a strongly immobilized component. At 15 °C from spectrum B of Figure 3, a value of $\tau_c \simeq 5 \times 10^{-5}$ s was estimated for the more immobilized component.

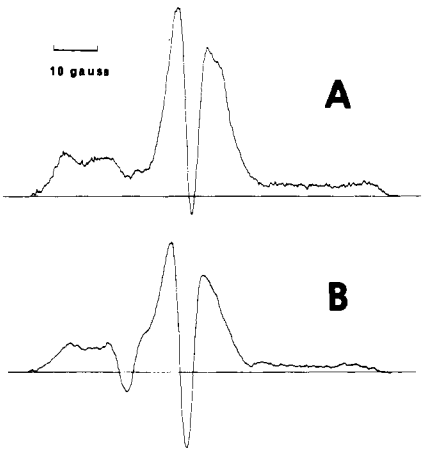


FIGURE 3: ST-ESR spectra in Ca^{2+}-ATPase–egg phosphatidylcholine complexes at 15 °C: (spectrum A) MSL; (spectrum B) (1,14)MSL.

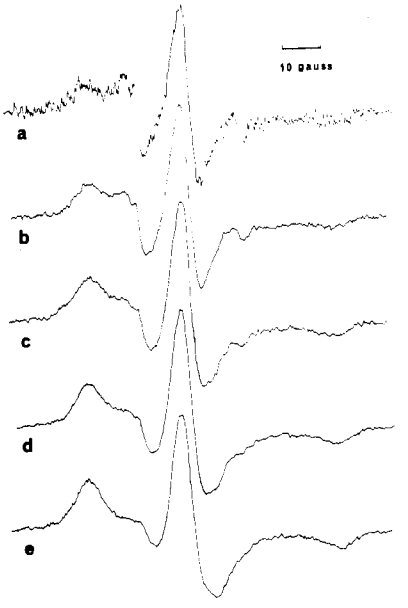


FIGURE 4: ESR difference spectra of the immobilized component in Ca^{2+}-ATPase–egg phosphatidylcholine complexes obtained by linear combinations of spectra corresponding to different temperatures: (A) 39 and 31 °C; (B) 31 and 23 °C; (C) 23 and 15 °C; (D) 15 and 7 °C; (E) 7 and −1 °C.

Quantitation of the Percentage of Immobilized Component. Two independent methods have been used. The first method involved the linear combination of two spectra recorded at slightly different temperatures with (1,14)MSL in the reconstituted system, for example, −1, and +7 °C or 7 and 15 °C, etc. The basic assumption of this method is that, to a first approximation, over a limited range of temperature only the ratio of the two components varies substantially, while the line shape of the components is only affected to a small extent. This method enabled us to obtain the approximate line shape of the immobilized component over a given range of temperature and thus to deduce the percentage of immobilized component. At each temperature (except for the low and high temperature, −1 and 39 °C), two different sets of results can be obtained. For example at 7 °C, the immobilized component is obtained either by combining spectra at 7 and −1 °C or 15 and 7 °C.

The set of immobilized components generated by the above procedure in reconstituted systems is given in Figure 4. The extreme splittings vary with temperature within a limited range, as already indicated (66–63 G). The immobilized spectrum obtained at high temperatures (spectrum A, Figure

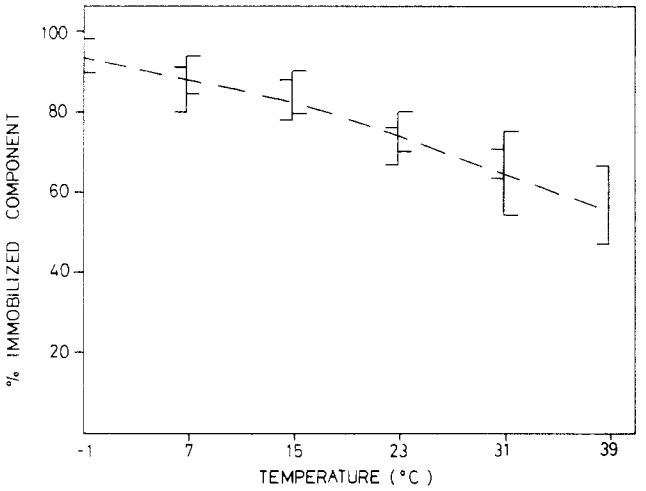


FIGURE 5: Percentage of immobilized component of (1,14)MSL bound to Ca^{2+}-ATPase in reconstituted vesicles, obtained by using the immobilized component of Figure 4. Error bars correspond to upper and lower limits for subtraction. The "arms" on the error bars of results obtained with the same "average immobilized component" point against each other.

4) contains a considerable level of noise. This is the result of the chemical instability of nitroxide at 39 °C in the presence of Ca^{2+}-ATPase. Partial reduction of the nitroxide takes place which preferentially affects the narrow component. This latter phenomenon has two consequences on the quantitation at 39 °C: (a) the immobilized component line shape is difficult to determine; (b) the percentage of immobilized component is overestimated.

The result of the quantitation of the spectral components by this procedure is given in Figure 5. The error bars correspond to lower and upper limits for subtraction (unambiguous appearance of only one component and phase inversion, respectively). It is seen from the figure that there is overlap between the results obtained with the two sets of evaluations at intermediary temperatures (7–31 °C). This confirms the validity of the method.[2]

The second method used to determine the percentage of immobilized component is independent of assumptions on temperature dependence of line shapes. It relies on the spectral modifications induced by the addition of the nonionic detergent $C_{12}E_8$ to the sample. We found that when $C_{12}E_8$ was added to reconstituted Ca^{2+}-ATPase vesicles under nonsolubilizing conditions, the spectral modifications can be accounted for by a change in the ratio of the two spectral components. This is illustrated in Figure 6A, where the spectra have been normalized to the same double integral. Isoclinic points are created by the superposition of the spectra corresponding to different amounts of detergent. An appropriate linear combination of two such spectra generates either the mobile component (spectrum B) or the immobile component (spectrum C).[3]

[2] It is interesting to note that because of the small variation of the hyperfine splitting of the "true" immobilized component, a reasonable approximation can be achieved by subtracting the same immobilized component at all temperatures. This latter operation does, however, slightly exaggerate the variation of the percentage of immobilized component with temperature, as pointed out by Brotherus et al. (1980).

[3] Spectra B and C were obtained by combining the spectrum without detergent with the spectrum obtained with 0.15 mg of detergent/mg of protein (i.e., under nonsolubilizing conditions). If the same operation is repeated by using the spectrum generated after solubilization (0.75 mg of detergent/mg of protein), the resulting spectra have some defects, indicating that the two-state approximation is no longer completely valid. This is probably because solubilization changes the line shape of the narrow component.

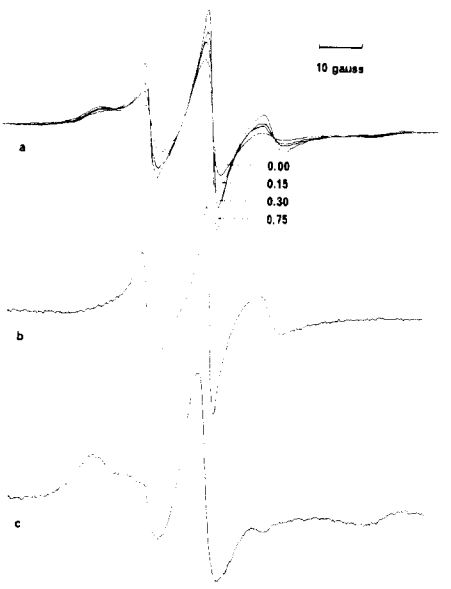


FIGURE 6: Effect of addition of $C_{12}E_8$ on ESR spectra of (1,14)MSL bound to Ca^{2+}-ATPase in Ca^{2+}-ATPase–egg phosphatidylcholine complexes at 15 °C. (a) Normalized spectra corresponding to increasing concentrations of $C_{12}E_8$ (expressed in milligrams of detergent per milligram of protein). Only 0.75 corresponds to solubilized membrane. (b) ESR difference spectrum of the fluid component at 15 °C, obtained from combination of spectra (a). (c) ESR difference spectrum of the immobilized component at 15 °C, obtained from combination of spectra (a).

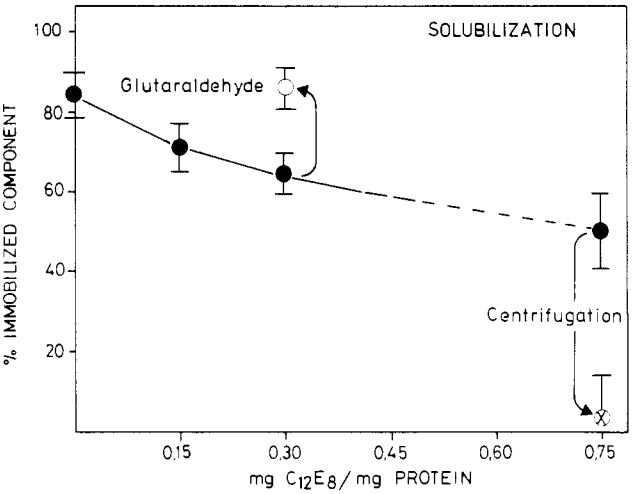


FIGURE 7: Percentage of immobilized component of (1,14)MSL bound to Ca^{2+}-ATPase in reconstituted vesicles as a function of detergent concentration (●). The solubilization starts to take place at the beginning of the broken line. The effects of glutaraldehyde (○) and of centrifugation (⊗) are also indicated. The temperature was 15 °C.

This operation was performed at various temperatures and enabled us to obtain another estimate of the percentage of immobilized component. The result (not shown) is practically superposable to the curve of Figure 5 with even smaller error bars. When reconstituted membranes were incubated overnight with 1% glutaraldehyde at 5 °C after the addition of a small amount of detergent (0.3 mg of $C_{12}E_8$/mg of protein), spectral analysis (Figure 7) showed that the percentage of immobilized component was greatly enhanced.

Both methods of spectral analysis were utilized also in the quantitation of the immobilized component in sarcoplasmic reticulum membranes. The results were very similar to those of the reconstituted system and in agreement with the data of Fellmann et al. (1980), based on a slightly different spin-label. The uncertainty, however, is larger than in the case of

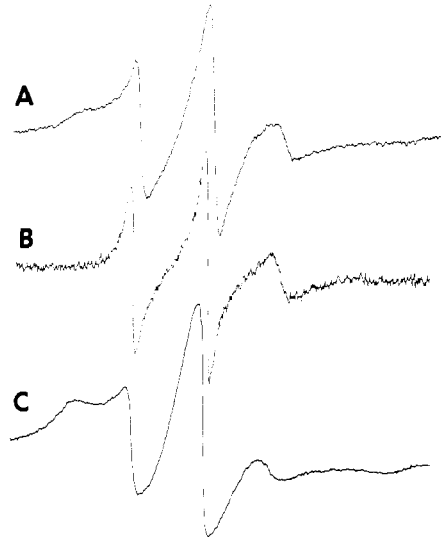


FIGURE 8: ESR spectra of (1,14)MSL bound to solubilized ATPase prepared by the DEAE-cellulose chromatographic procedure described under Materials and Methods. The temperature was 20 °C. (A) Solubilized ATPase before centrifugation. (B) Supernatant after centrifugation for 40 min at 130000*g* on the Beckman airfuge. (C) Pellet resulting from the centrifugation, resuspended in the elution buffer.

the reconstituted systems, obviously because of the residual unbound labels.

Solubilized Proteins. As mentioned in the previous paragraph, and illustrated on Figures 6 and 7, addition of a small amount of $C_{12}E_8$ decreases the percentage of immobilized component. This is not related to partial solubilization as judged from centrifugation assays. Furthermore, after solubilization, which takes place at above ~0.5 mg of detergent/mg of protein, the percentage of immobilized component is still large (~50%; see Figure 7). This is also the case for solubilized Ca^{2+}-ATPase prepared by the DEAE-cellulose chromatographic procedure described in the experimental section (Figure 8A). However, after centrifugation for 40 min at 130000*g* on the Beckman airfuge, almost no strongly immobilized component can be seen in the spectrum of the supernatant (Figure 8B). Only a slight restriction of the probe motion is present in this case. The supernatant spectrum is broader than the spectrum of unbound label, which under the same conditions gives rise to very narrow lines (not shown). Analytical ultracentrifugation was performed on the samples corresponding to Figure 8A,B. Most of the protein (~65%) in the centrifuged specimen (Figure 8B) sedimented with a sedimentation coefficient of 5.2 S which corresponds to the value of a monomer of ATPase (le Maire et al., 1978). The remaining part had a sedimentation coefficient corresponding approximately to that of a dimer. Thus, the spectrum of Figure 8B corresponds to 65% monomers and 35% dimers. The line shape of spectrum B indicates principaly a mobile component which can be associated with the monomeric form of the ATPase molecule. Spectrum B may contain also a broad component corresponding to a restricted motion and comprising 10–20% of the total intensity. Thus, a possible conclusion is that the monomer is associated with an ESR spectrum with exclusively narrow lines, while the dimer gives rise to a mixture of a narrow component and a broad component.

The uncentrifuged system (Figure 8A) in addition to the monomer and the dimer had a sedimentation profile with a broad boundary, suggesting the presence of a heterogeneous component comprising ~80% of the sedimenting material. The average value for the sedimentation coefficient of this additional component was 13–15 S. Thus, the strongly immobilized component in the ESR spectrum (Figure 8A) seems to be associated with ATPase complexes of relatively large size (trimers/tetramers and larger).

The spectrum of the pellet resulting from centrifugation on the airfuge is shown in Figure 8C. It can be seen that the immobilized component is more pronounced in this sediment than in the original specimen (Figure 8A). This is probably due to the partial physical separation of large oligomers from monomers and dimers. Also, further aggregation may have been induced by the tight packing of the protein at the bottom of the centrifuge tube, since the pellet formed a turbid suspension, in contrast to the original sample, which was transparent.

Localization of Probe by Ascorbate Assay. The reduction rate of the ESR signal due to chemical reduction by ascorbate of (1,14)MSL was studied in order to obtain some information on the localization of the nitroxide in the membrane. The peak heights of the immobilized and more mobile component were monitored separately by using reconstituted ATPase membranes (Figure 9A) and ATPase solubilized in $C_{12}E_8$ (Figure 9B). In the first case it can be seen that the reduction of (1,14)MSL is very slow for both components and quite similar to that of the noncovalently bound 16-doxylstearic ester which is deeply buried in the hydrophobic core of the membrane. This may be compared to the much faster reduction of a 5-doxylstearic ester (Figure 9A), which presumably has the nitroxide located nearer to the hydrophilic phospholipid head groups. In detergent the nitroxide of the (1,14)MSL bound to the ATPase is more accessible to reduction than in the membrane, although not nearly to the same extent as the free fatty acid label in buffer without detergent micelles (Figure 9B). Finally, it may be noted that the immobilized component is reduced at a slightly slower rate than the mobile component.

Discussion

Ca^{2+}-ATPase is known to possess several sulfhydryl groups, both in the hydrophilic and hydrophobic moiety (Allen & Green, 1978), which are potential reaction sites for a maleimide spin-label. Because of the hydrophobic character of (1,14)MSL and the relatively slow reaction rate with Ca^{2+}-ATPase, it seems likely that during and after covalent coupling to Ca^{2+}-ATPase the spin-label is positioned within the hydrophobic interior of the membrane. This is a prerequisite for any discussion on lipid–protein or protein–protein interactions in Ca^{2+}-ATPase membranes based on the use of such covalently attached spin-labels. The hydrophobic character of the nitroxide environment was confirmed by the ascorbate reduction assay which showed a slow reduction rate compatible with the nitroxide being deeply buried in the membrane. Published studies of ascorbate reduction of the water-soluble short-chain maleimide (MSL) bound to Ca^{2+}-ATPase have shown the rate of reduction to be much higher (Tonomura & Morales, 1974; Champeil et al., 1980). Furthermore, we have found that it is possible to bind MSL to thiol-containing Sepharose 4B material, whereas (1,14)MSL does not react with such a hydrophilic matrix.

Origin of the Two Spectral Components Observed with Ca^{2+}-ATPase. First Hypothesis: Difference between First and Second Layer. One explanation could be that the probe on the covalently attached chain partitions between two lipid layers around the protein hydrophobic interface. The immobilized component would correspond to the immobilized "annulus", which according to Metcalfe & Warren (1977) covers Ca^{2+}-ATPase. The mobile component would represent

the bulk lipid phase viscosity. In order to be consistent with the data, this hypothesis requires that the partition coefficient must be more in favor of the bulk lipids as temperature is increased or detergent is added.

The main objection to this interpretation is that the immobilization observed here is characterized by a very long correlation time ($\sim 10^{-5}$–10^{-4} s). This is incompatible with the recent ^2H NMR data of Oldfield and co-workers (Kang et al., 1979; Rice et al., 1979). An immobilized lipid annulus with such a long lifetime would be visible on the time scale of ^2H NMR. Furthermore, according to the work of Knowles et al. (1979) and Watts et al. (1980), the immobilized lipids surrounding an intrinsic protein such as cytochrome oxidase or rhodopsin would be characterized by correlation times on the order of 10^{-7}–10^{-8} s. Since this is 3 orders of magnitude faster than what we observed with (1,14)MSL bound to Ca^{2+}-ATPase, the hypothesis of immobilized lipid chains in a boundary layer appears inconsistent with the data.

Second Hypothesis: *Different Aggregational States of Ca^{2+}-ATPase.* On the basis of the data presented in this paper, we consider that the most likely origin of the strongly immobilized component is the trapping of spin-label between Ca^{2+}-ATPase peptides. This is suggested by the observation that (a) the addition of glutaraldehyde increased the amount of immobilized component and (b) ultracentrifugation of solubilized Ca^{2+}-ATPase indicated that strong immobilization was only observed if the protein was in oligomeric form. Similar results have been reported with cytochrome oxidase by Swanson et al. (1980), who found that the immobilized component of covalently attached fatty acid spin-label was present only in aggregated protein. By contrast, it is very striking that the same probe does not give rise to a strongly immobilized component when attached to rhodopsin, which probably occurs as a monomer in the membrane. This latter conclusion was deduced from chemical cross-linking and energy-transfer experiments (Ebrey, 1971), from the fast rotational diffusion of rhodopsin (Cone, 1972), and from X-ray measurements of the average distance between rhodopsin molecules (Chabre, 1975).

As mentioned in the introduction, various experimental approaches have suggested the presence of oligomeric complexes of Ca^{2+}-ATPase in the membrane. Trapping of spin-label by Ca^{2+}-ATPase could be caused by insertion into such complexes or possibly by the formation of clusters of complexes. None of these suggestions is mutually exclusive, especially at low temperature where immobilization is nearly complete. The mobile component most probably represents spin-labels in contact with both protein and the lipid bilayer. It could represent either the exterior face of protein oligomers or monomeric Ca^{2+}-ATPase, coexisting with oligomers in the membrane. According to the latter suggestion, an increase in temperature or the presence of $C_{12}E_8$ would result in increased dissociation of oligomeric Ca^{2+}-ATPase into monomers. This possibility is currently being subjected to further examination by other techniques.

At very low temperature (0 °C), however, it is possible that the exchange rate becomes slow enough so as to permit the separation of two spectral components corresponding to the first and second lipid layer surrounding the protein. This means that part of the immobilized component may be due to this temperature-dependent exchange effect; as a consequence, the temperature-dependent oligomerization would be minimized.

The present results concerning the effect of temperature are not in agreement with the model for oligomerization of Ca^{2+}-ATPase presented by Hoffmann et al. (1979), according to which temperature increase produces an increased oligomerization. This was deduced from measurements of rotational correlation times by the flash photolysis method. However, Cherry's measurements of rotational correlation times seem to be in favor of a dissociation of Ca^{2+}-ATPase at high temperature (Cherry, 1979; Bürkli & Cherry, 1981). This is indirect evidence, since absolute values of τ_c do not permit a conclusion as to whether or not membrane proteins are oligomers or monomers.

In our work it was important to obtain an estimate of the protein rotational correlation time in the reconstituted systems in order to check that the proteins did not form large artificial aggregates.[4] Because of this control and the freeze–fracture results (see Materials and Methods), we are confident that the protein–protein interactions observed by ESR with Ca^{2+}-ATPase in reconstituted vesicles are physiologically relevant.

Third Hypothesis: *The Two Spectral Components Are Associated with Different Conformations of Ca^{2+}-ATPase.* Various investigators have suggested that Ca^{2+}-ATPase undergoes a conformational change at a temperature close to 20 °C (Dean & Tanford, 1978; Anzai et al., 1978; Kirino et al., 1978; Hoffmann et al., 1979). Considering the magnitude of the error bars in Figure 5, it is possible to draw a curve representing the percentage of immobilized component with a sharp bend around 20 °C. Therefore, it is conceivable to associate the two spectral components with two conformations of Ca^{2+}-ATPase, if it is assumed that the conformational change leads to a change in the proportion of immobile and mobile label. Such a view has been advanced by Hoffmann et al. (1980) in the case of bacteriorhodopsin.

We wish to point out that a scheme of this kind is not inconsistent with the second hypothesis. We have previously presented evidence that a dissociation of detergent-solubilized oligomeric Ca^{2+}-ATPase into monomers involves a conformational change (Andersen et al., 1980). A similar conformational change induced by an increase in temperature or addition of nonsolubilizing amounts of detergent to the membrane may lead to changes of protein–protein interactions and to an increase in the amount of monomer in the membrane.

About the Shape of Mobile Component. The mobile component obtained after computer subtraction of a strongly immobilized component (see Figure 6B) is quite similar to the type of spectrum observed with (1,14)MSL bound to rhodopsin in egg lecithin (Davoust et al., 1979). In terms of correlation time it corresponds to a motion of a few nanoseconds. The spectrum indicates some restriction in the mobility of the probe at the boundary since a free fatty acid would give narrower lines at the same temperature. From spectrum B in Figure 6 one could subtract a component corresponding to a "motionally restricted probe", with a splitting on the order of 55 G. This is the technique utilized by the group of D. Marsh (Knowles et al., 1979; Watts et al., 1979). Such a splitting would correspond to correlation times of a few tens of nanoseconds. This is not fundamentally different from what we find. Furthermore, computer simulation of rapid chemical exchange between a "strongly immobilized signal" and a free fatty acid type of signal gives exactly the shape observed in Figure 6B (J. Davoust and P. F. Devaux, unpublished results).

[4] In the spin-label study of Hesketh et al. (1976), there is no step of solubilization and dialysis after the lipid exchange. Furthermore, the lipid to protein ratio in their lipid-substituted Ca^{2+}-ATPase preparations was low, raising the possibility that the immobilized component observed in their studies arose from artificially produced protein aggregates.

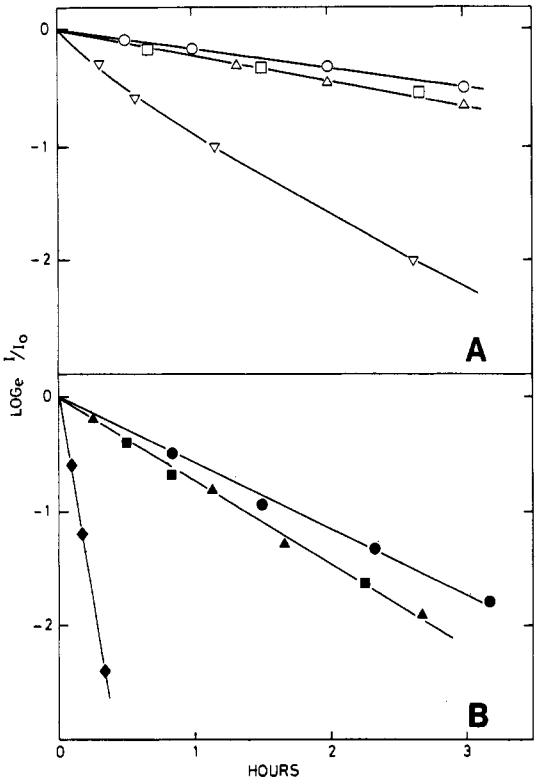


FIGURE 9: Ascorbate reduction assay of (1,14)MSL bound to reconstituted ATPase membranes (A) and detergent-solubilized ATPase (B). The signal intensities (I) in the low-field region were monitored at various times after addition of 5 mM ascorbate at 5 °C as described under Materials and Methods. (○, ●) Immobilized component of (1,14)MSL; (□, ■) mobile component of (1, 14)MSL; (△, ▲) 16-doxylstearic ester; open symbols correspond to ATPase membranes, filled to solubilized ATPase; (▽) 5-doxylstearic ester bound to ATPase membranes; (♦) free 16-doxylstearic acid in buffer without detergent.

Therefore, it may be considered as reflecting the average mobility of a chain, forced to stay at a protein–lipid interface, where rapid exchange between the first lipid shell and the bulk lipids occurs.

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Molecular Symmetry and Metastable States of Enzymes Exhibiting Half-of-the-Sites Reactivity†

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ABSTRACT: Half-of-the-sites reactivity in oligomeric enzymes has generally been accepted as evidence for structural asymmetry between subunits. However, we show that the symmetric two-state allosteric model [Monod, J., Wyman, J., & Changeux, J.-P. (1965) *J. Mol. Biol. 12*, 88–118] is quantitatively consistent with half-of-the-sites reactivity data for several hexameric and tetrameric enzymes. Specifically, the time courses for both the modification and the inactivation of glutamate dehydrogenase by glutamyl α-chloromethyl ketone and uridine diphosphoglucose dehydrogenase by 5-(iodoacetamidoethyl)aminonaphthalene-1-sulfonic acid are fit with just five parameters for each enzyme–modifier pair. In the case of glyceraldehyde-3-phosphate dehydrogenase, the time courses for modification of the yeast enzyme by iodoacetic acid and the rabbit-muscle enzyme by 3,3,3-trifluorobromoacetone are fit with the same model, and parameter values from these fits are used to generate theoretical inactivation curves which are found to agree well with the experimentally measured inactivation. We conclude that half-of-the-sites reactivity, if it is not an artifact of residual heterogeneity, could be a kinetic phenomenon related to metastability of partially modified states of a symmetric oligomer and that asymmetry between subunits should therefore not necessarily be inferred from such behavior. If similar metastability occurs in substrate binding, it may play a significant role in mechanisms of catalysis and control. In such cases, the virtual inaccessibility of the substrate binding equilibrium would preclude conventional quasi-equilibrium models for the enzyme kinetics.

The term "half-of-the-sites reactivity" encompasses several different phenomena observed in some oligomeric enzymes. One of these is biphasic kinetics for ligand binding or active site modification. For example, in the alkylation of yeast glyceraldehyde-3-phosphate dehydrogenase with iodoacetate or iodoacetamide, the first 2 equiv react much faster than the third and fourth (Stallcup & Koshland, 1973a,b). A biphasic time course is also observed for the incorporation of radioactivity from tritiated L-glutamyl α-chloromethyl ketone into the six subunits of bovine liver glutamate dehydrogenase (Rasool et al., 1976).

In extreme cases, the last reaction steps are so slow that complete ligand association or chemical modification is not observed at all. For example, in the absence of carbamoyl phosphate, succinate has been found to bind to only three of the six catalytic subunits of aspartate transcarbamoylase from *Escherichia coli* (Suter & Rosenbusch, 1976). Similarly, the maximum number of molecules of the inducer isopropyl β-D-thiogalactoside bound per tetramer of the *lac* repressor of *E. coli* is found to range from 2.3 to 4.0 depending on the pH, temperature, and anti-inducer concentration (Oshima et al., 1974).

Another facet of half-of-the-sites reactivity is the observation that some enzymes may be rendered completely inactive by modification of only half of the subunits. For example, rabbit-muscle glyceraldehyde-3-phosphate dehydrogenase shows no catalytic activity when modified by β-(2-furyl)acryloyl phosphate at only two of the four subunits (MacQuarrie & Bernhard, 1971). The yeast enzyme shows no catalytic activity when modified by *p,p'*-difluoro-*m,m'*-dinitrophenyl sulfone at only two of the four subunits (Givol, 1969). Similarly, the activity of cytidine triphosphate synthetase is abolished by the binding of 6-diazo-5-oxonorleucine to only two of the four subunits (Levitzki et al., 1971).

The functional significance of half-of-the-sites reactivity remains unclear, and attention has focused on its structural implications [for a summary see Levitzki (1978)]. It is generally accepted that half-of-the-sites reactivity requires asymmetry among the enzyme subunits. The concerted allosteric mechanism of Monod et al. (1965) has been excluded because its symmetric character is known to produce only positive cooperativity in homotropic ligand binding *equilibria*. In the case of yeast glyceraldehyde-3-phosphate dehydrogenase, it has been shown that the apparent asymmetry does not preexist chemical modification and it was therefore inferred that the presumed asymmetry is allosterically induced by chemical

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