A Spin-Label Study of Energy-Coupled Active Transport in *Escherichia coli* Membrane Vesicles†

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ABSTRACT: Electron paramagnetic resonance (epr) signals from *Escherichia coli* ML 308-225 membrane vesicles spin-labeled with *N*-oxyl-4′,4′-dimethyloxazolidine derivatives of stearic acid decrease according to pseudo-first-order kinetics upon the addition of electron donors. The rate of the decrease is dependent upon the added electron donor, D-lactate or succinate. The rate of epr signal reduction after D-lactate addition is twice that of succinate. The position of the nitroxyl group along the fatty acid chain also affects the rate; the rate for a nitroxyl group which is five carbon atoms [I(12,3)] away from the carboxyl group is greater than that for a nitroxyl which is 12 carbon atoms [I(5,10)] away, and still greater than that for a nitroxyl which is 16 carbon atoms [I(1,14)] away. Sulfhydryl reagents (diazinedicarboxylic acid bisdimethylamide, *N*-ethylmaleimide, or *p*-hydroxymercuribenzoate) inhibit the loss of epr signal intensity. The effect of *p*-hydroxymercuribenzoate is reversed by dithiothreitol. Inhibitors of the electron transfer chain (2-heptyl-4-hydroxyquinoline *N*-oxide or potassium cyanide) do not inhibit the reduction of spin-labels when added to the membrane vesicles. Competitive inhibitors of D-lactate dehydrogenase, oxalic acid or oxamic acid, reduce the rate of spin-label reduction when D-lactate is used as the electron donor, but these reagents have no effect on the reduction when succinate is used. These results suggest that certain sulfhydryl groups in the electron transfer chain are involved in the reduction of spin-labeled stearic acids and that these sulfhydryls are coupled to the respiratory chain between the flavine-linked dehydrogenase and cytochrome b_1.

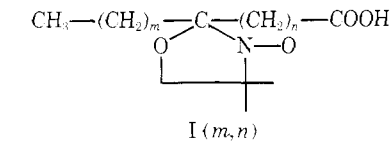
Recent observations by Kaback and coworkers have shown that bacterial cytoplasmic membrane vesicles can be used as an excellent model system to investigate active transport (Kaback, 1972, 1974). In *Escherichia coli* membrane vesicles, the respiration-linked transport system is coupled primarily to the oxidation of D-lactate to pyruvate, catalyzed by a flavine-linked, membrane-bound D-lactate dehydrogenase (Kaback, 1972). Other oxidizable substrates, such as succinate, L-lactate, and NADH, are less efficient as electron donors in support of active transport in *E. coli*. On the basis of various inhibitor studies evidence has been presented which demonstrates that the site of energy coupling is located between the flavine-linked dehydrogenase and cytochrome b_1 (Kaback, 1972; Kaback and Hong, 1973).

The spin-label technique has been very useful in the study of the structure-function relationships in biological systems (for a review on this subject, see McConnell and McFarland, 1970). In particular, the introduction of stable nitroxide free radicals into model and biological membranes has yielded a great amount of information concerning membrane structure (Seelig, 1971; Hubbell and McConnell, 1971; Keith *et al.*, 1973). Studies in membranes have focused on the spectral relationships of motion of the label relative to its environment and on the intricate involvement of lipids and membrane functions. More recently, spin-label studies have been carried out to investigate the effects of poly(amino acids) and proteins on the structure of model and biological membranes (Yu *et al.*, 1974; Rottem and Samuni, 1973; Electr *et al.*, 1973; Hong and Hubbell, 1972; Jost *et al.*, 1973).

In this communication, we report a spin-label study of energy coupling of active transport in *E. coli* membrane vesicles.

Experimental Section

Materials. Cells of *E. coli* ML 308-225, a gift of Dr. H. R. Kaback, were grown at 37° on minimal medium A (Davis and Mingioli, 1950) supplemented with 1% glycerol or sodium succinate and harvested in mid-log growth. [^{14}C]-L-Proline was purchased from New England Nuclear Co. Lysozyme, DNase, RNase, lithium salt of D-lactate, oxamic acid, *N*-ethylmaleimide, and *p*-hydroxymercuribenzoate were all purchased from Sigma. Oxalic acid was purchased from Fisher. Iodoacetamide was obtained from K & K Laboratories. Amobarbital (amytal) and diazinedicarboxylic acid bisdimethylamide (diamide) were gifts of Dr. H. R. Kaback. Dithiothreitol was obtained from Calbiochem. Spin-labeled stearic acids of the general formula were obtained from Synvar Associates.



I(*m*,*n*)

Membrane vesicles of *E. coli* ML 308-225 were prepared as described by Kaback (1971) with the following modifications: cells were not washed after harvesting; only mild homogenization in the presence of 5-7 mg of RNase and of DNase was used; the vesicles were pelleted once at 46,000*g* and resuspended to a concentration of 30 mg of membrane protein/ml in 0.1 M potassium phosphate buffer

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(pH 6.6).

Methods. TRANSPORT ASSAYS. Cytoplasmic membrane vesicles were diluted to a final concentration of 0.1 mg of protein/ml, preincubated for 4 min at 30°, and aerated with a magnetic stirrer. [1-^{14}C]Proline (S.A. 220 Ci/mol) and D-lactate were added to the preincubated cells to final concentrations of 10–15 μM and 20 mM respectively; 100-μl aliquots were taken from the reaction vessel and transferred to a suction apparatus where each was filtered through a 0.22-μ GSWP Millipore filter. Each filter was washed with 10 ml of 0.1 M lithium chloride and counted in a Packard liquid scintillation counter.

Membrane protein concentration was estimated spectrophotometrically with an optical density of 1.5 at 600 nm (1-cm cuvet) as equal to 10 mg of membrane protein/ml (Kaback, 1971).

All epr spectra were obtained from a Varian model E-4 X-band spectrometer equipped with a variable temperature controller set at 30°.

Membrane vesicles were labeled with *N*-oxyl-4′,4′-dimethyloxazolidine derivatives of stearic acid [I(12,3), I(5,10), or I(1,14)]. The spin-labels were dissolved in ethanol and evaporated to dryness by passage of a stream of dry nitrogen; 100 μl of membrane suspension (20 mg of protein/ml) in 10 mM potassium phosphate (pH 6.6) and 10 mM $MgSO_4$ was added to a tube containing the dry precipitate of spin-label (final concentration 10^{-4}–10^{-5} M). The vesicles were vortexed and pipetted into a 50-μl capillary tube which was placed in a Varian E-4 cavity. The absence of a free spin-label peak in the resulting epr spectra indicates total incorporation of label.

The values of R (as given in Figures 1–4) were calculated from the ratio $h(t)/h(0)$. The values of h, the peak to peak height of the low field line ($m = 1$), were determined at time equal to 0 ($t = 0$) and at time intervals (t = t) during the reaction. Since there was no observed change in the line widths during the reaction, $h(t)$ is proportional to the concentration of nitroxide radical at time t and R $[h(t)/h(0)]$ is equal to the fraction of free radical remaining at time t. Initially all three hyperfine lines ($m = 1,0,-1$) were used to determine R and found to be equivalent.

Results

1. Kinetics of the Reduction of Spin-Labels. The rates of reduction of spin-labeled stearic acids incorporated into membrane vesicles isolated from glycerol grown *E. coli* ML 308-225 cells follow first-order kinetics. The pseudo-first-order rate constants depend on temperature and the nature as well as concentration of electron donors. In the absence of D-lactate the epr signal intensity of spin-label I(1,14) is stable for at least 30 min at 30°. Furthermore, D-lactate alone does not affect the signal intensity. The loss of signal intensity, therefore, depends on some membrane component(s) as well as on added electron donor. Since nitroxyl radicals can be easily reduced by a number of reducing agents, the loss of intensity is most likely due to a reduction of the spin-label.

Typical pseudo-first-order rate constants for the reduction of spin-label I(1,14) are 0.61 and 0.26 min^{-1} in the presence of 20 mM D-lactate and 20 mM sodium succinate, respectively. The pseudo-first-order rate constants for the reduction of spin-labels can vary up to a factor of 2 depending on preparation conditions, storage, and age of the vesicles. Succinate also stimulates the loss of epr signal intensity of the spin-labels, but at a rate less than one-half that of D-lactate when compared on membrane vesicles prepared and stored under identical conditions. The order of the effectiveness, D-lactate > succinate, is in agreement with the ability of these substrates to stimulate transport (Kaback, 1972).

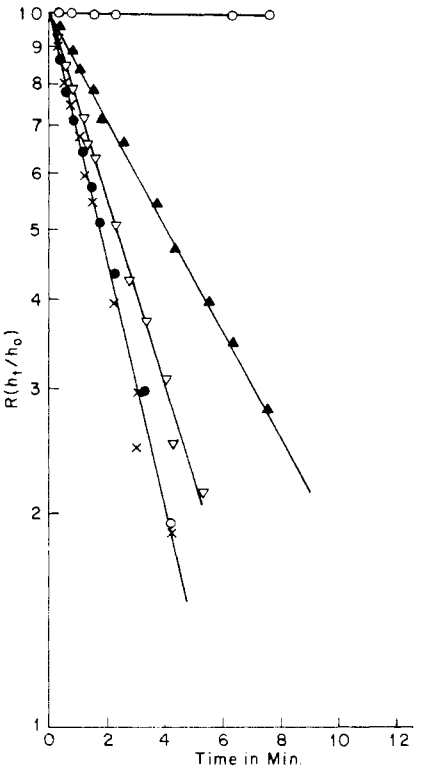

FIGURE 1: Inhibition of the reduction of spin-labeled stearic acid I(1,14) by electron transfer chain inhibitors. Aliquots of *E. coli* ML 308-225 membrane vesicles in 10 mM potassium phosphate (pH 6.6) and 10 mM magnesium sulfate were spin-labeled as described in the Experimental Section. Respiratory inhibitors and lithium D-lactate were added to the following final concentrations: (○) no addition of D-lactate (control); (▽) 20 mM D-lactate; (●) 20 mM D-lactate + 100 mM KCN; (x) 20 mM D-lactate + 10^{-4} M 2-heptyl-4-hydroxyquinoline N-oxide; (▲) 20 mM D-lactate + 10 mM amobarbital. The samples were recorded at 30° and the epr signal intensity ratios (R) were calculated as described in the Experimental Section.

2. Effects of Electron Transfer Inhibitors. Electron transfer inhibitors, HOQNO[1] and KCN, do not inhibit the reduction of spin-label (Figure 1). Concentrations which have been shown to profoundly inhibit respiration and transport (Barnes and Kaback, 1971; Lombardi and Kaback, 1972) have little effect on the reduction of the nitroxyl radical. In fact, in the presence of HOQNO or KCN, there is a slight increase in the reduction rate. Cox *et al.* (1970) have shown that these inhibitors act on the main electron transfer chain after cytochrome b_1. The increase in the rate of spin-label reduction in the presence of either HOQNO or KCN is likely due to an inhibition of some respiratory "intermediates" which compete with spin-labels for electrons. In the presence of amytal there is a slight decrease in the rate of reduction of spin-label I(1,14) (Figure 1). Amytal is known to inhibit a flavoprotein between D-lactate dehydrogenase and cytochrome b_1 (Cox *et al.*, 1970).

[1] Abbreviations used are: MalNEt, *N*-ethylmaleimide; HgBzOH, *p*-hydroxymercuribenzoate; amytal, amobarbital; diamide, diazinedicarboxylic acid bisdimethylamide; HOQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide.

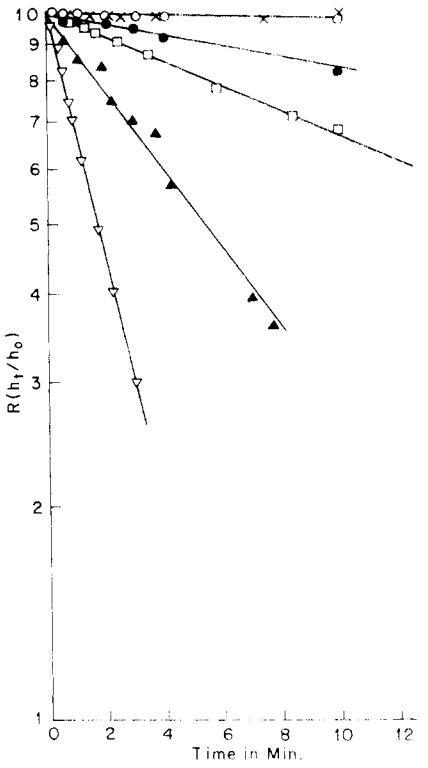

FIGURE 2: Effects of sulfhydryl reagents on the reduction of spin-labeled stearic acid I(1,14); 100-μl aliquots of *E. coli* ML 308-225 membrane vesicles (20 mg of membrane protein) in 10 mM potassium phosphate (pH 6.6) and 10 mM magnesium sulfate were preincubated for approximately 20 min at 4° with sulfhydryl reagents at final concentrations: 2 mM N-ethylmaleimide (MalNEt), 10 mM diazinedicarboxylic acid bisdimeth lamide (diamide), 5 mM *p*-hydroxymercuribenzoate. After preincubation the vesicles were spin-labeled and lithium D-lactate was added (where indicated) at final concentration of 20 mM; 5 mM dithiothreitol (final concentration) was added to the sample marked HgBzOH + dithiothreitol. The spectra were recorded at 30°: (○) no addition of D-lactate (control); (▽) D-lactate; (●) D-lactate + MalNEt; (□) D-lactate + diamide; (x) D-lactate + HgBzOH; (▲) D-lactate + HgBzOH + dithiothreitol.

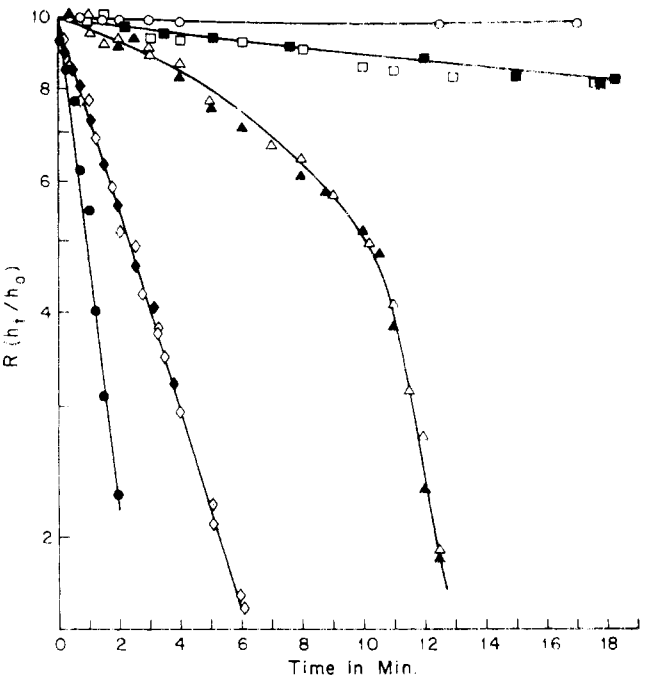

FIGURE 3: Effects of oxalic and oxamic acids on the reduction of spin-labeled stearic acid I(1,14). Membrane vesicles in 10 mM potassium phosphate (pH 6.6) and 10 mM magnesium sulfate were spin-labeled as described in the Experimental Section. Oxalic or oxamic acids in 0.1 M potassium phosphate (titrated with 2 N KOH to pH 6.6) were added at final concentrations of 20 mM. The effect of the order of addition on the apparent first-order kinetic plot was studied by either preincubating the vesicles for 2 min at either 30° or 4° with oxalic or oxamic acid and then adding D-lactate (final concentration 20 mM) at zero time; or adding D-lactate first and at zero time adding oxamic or oxalic acid. The spectra were then recorded at 30°: (○) no addition of D-lactate (control); (●) 20 mM D-lactate; (△) 20 mM D-lactate + 20 mM oxamic acid; (▲) 20 mM oxamic acid (preincubated 2 min at 30°) + 20 mM D-lactate; (□) 20 mM D-lactate + 20 mM oxalic acid; (■) 20 mM oxalic acid (preincubated 2 min at 30°) + 20 mM D-lactate; (◇) 20 mM succinate; and (◆) 20 mM oxalic acid (preincubated 2 min at 30°) + 20 mM succinate.

3. Effects of Sulfhydryl Reagents. As shown in Figure 2, D-lactate dependent reduction of spin-label I(1,14) is sensitive to the sulfhydryl reagents, HgBzOH, MalNEt, or diamide. Both HgBzOH and MalNEt have been found to inhibit both D-lactate oxidation and D-lactate dependent transport in membrane vesicles (Kaback and Barnes, 1971; Kaback and Hong, 1973). The fact that the sulfhydryl reagents significantly decrease the rate of reduction of spin-labeled stearic acids indicates that the reduction of the nitroxyl groups is dependent on certain SH group(s) in the membrane vesicles. Dithiothreitol is known to reverse the inhibitory effect of *p*-chloromercuribenzoate on the initial rate of amino acid uptake (Kaback and Barnes, 1971). The addition of dithiothreitol reverses the effect of HgBzOH so that the rate of reduction is almost equal to that in the absence of this SH reagent.

4. Effects of Oxamic and Oxalic Acids. Figure 3 shows the rates of reduction of spin-label I(1,14) in the presence of oxalic and oxamic acids, both competitive inhibitors of D-lactate dehydrogenase (Barnes and Kaback, 1971; Kohn and Kaback, 1973). Oxalic acid at 20 mM essentially blocks the reduction of the spin-label if D-lactate is the electron donor, but has no effect on succinate dependent reduction. Furthermore, the inhibition of spin-label reduction is independent of the order of addition of D-lactate and oxalic acid to the membrane vesicles (Figure 3). Oxamic acid at 20 mM markedly decreased the initial rate of D-lactate dependent reduction of the spin-label, but after ~10 min, the rate of reduction is the same as that in the absence of oxamate (Figure 3). Again, as with oxalic acid, this effect does not depend on the sequence of addition of oxamic acid and D-lactate (Figure 3). The fact that the order of addition of oxalate (or oxamate) and D-lactate to the vesicles has no effect on the rate of reduction of the spin-label suggests that the effect of oxalate (or oxamate) on the spin-label reduction is not due to the prevention of D-lactate's entry into membrane vesicles by these inhibitors.

5. Rates of Reduction as a Function of the Position of the Nitroxyl Group Along the Fatty Acid Chain. The rate of reduction of spin-labels I(1,14), I(5,10), and I(12,3) in membrane vesicles is shown in Figure 4. The rate depends on the position of the nitroxyl groups along the fatty acid chain. These follow the order: spin-label I(12,3) > spin-label I(5,10) > spin-label I(1,14). These results suggest that the rates of reduction depend on how deep into the lipid bilayer the nitroxyl group transverses. Those nitroxyl groups which penetrate deeper into the bilayer have slower rates of reduction.

6. Transport of Proline in the Presence of Spin-Label I(1,14). The presence of spin-label I(1,14) at 10^{-4}–10^{-5} M in membrane vesicles has little effect on the steady-state accumulation of [^{14}C]proline. This suggests that the spin-la-

beled membrane vesicles possess transport activities and are not significantly perturbed by the addition of spin-labeled fatty acids. In the presence of KCN, both spin-labeled and unlabeled membrane vesicles do not significantly take up proline (data not shown).

Discussion

The data presented in this study demonstrate that the rates of reduction of *N*-oxyl-4′,4′-dimethyloxazolidine derivatives of stearic acid incorporated into *E. coli* membrane vesicles are dependent upon the nature of electron donors, sulfhydryl reagents, and electron transfer inhibitors. The respiratory inhibitors, KCN and HOQNO, which Cox *et al.* (1970) have shown to affect the respiratory chain after cytochrome b_1 do not inhibit the reduction of the spin-labels in the membrane vesicles. Amytal, which acts on a flavoprotein between D-lactate dehydrogenase and cytochrome b_1 (Cox *et al.*, 1971), produces slight inhibition of nitroxyl reduction. These findings place the site of coupling of spin-labeled stearic acids in the electron transfer chain before cytochrome b_1. Oxalic acid, a potent competitive inhibitor of D-lactate dehydrogenase, decreases the rate of reduction if D-lactate is used as the electron donor. Oxalic acid has no effect on the spin-label reduction if succinate is used to supply electrons to the respiratory chain. Sulfhydryl reagents (HgBzOH, MalNEt, or diamide) can prevent the reduction of the spin-labels. The effects of MalNEt and HgBzOH on D-lactate oxidation and transport are not exerted at the level of the primary dehydrogenase (Kaback and Hong, 1973). On the other hand, diamide inhibits proline and lactose transport in *E. coli* membrane vesicles, but does not affect D-lactate oxidation (Kaback and Hong, 1973). Furthermore, Kohn and Kaback (1973) found that *P*-chloromercuribenzonate, *N*-ethylmaleimide, and iodoacetate in concentrations as high as 10 mM did not affect purified D-lactate dehydrogenase as measured by dichlorophenolindophenol or tetraozolium dye reduction. These results suggest that certain sulfhydryl groups (such as those which react with diamide) in the respiratory chain play a role in the reduction of spin-labeled stearic acids and that these sulfhydryl groups are coupled to the respiratory chain between the flavine-linked dehydrogenase and cytochrome b_1.

The dependence of the spin-label reduction on electron donors and on sulfhydryl reagents can be explained in the following two ways. First, electrons from an electron transfer intermediate which is in the respiratory chain could directly reduce the nitroxyl groups of the spin-labeled stearic acids. This electron transfer intermediate, however, could be part of a shunt which couples D-lactate oxidation to transport. In this scheme the sulfhydryl groups alkylated by the thiol reagents could be in the electron transfer chain prior to this intermediate. Secondly, the spin-labeled stearic acids could be reduced directly by certain sulfhydryl groups located in the respiratory chain. Morrisett and Drott (1969) have shown that nitroxyl radicals can be reduced by SH groups. As was discussed in the Result section, the spin-labeled stearic acids incorporated into membrane vesicles are quite stable in the absence of an electron donor. A likely role of D-lactate or succinate oxidation would be to supply energy to allow for conformational changes in one or more respiratory-linked proteins which contains these SH groups. In this way, a conformational change in the respiratory-linked protein(s) when electron donors are added would expose these SH groups for reaction with the nitroxyl groups of the labeled stearic acids. Schuldiner *et al.* (1974) have recently reported that certain dansyl galactosides can bind to β-galactosidase carrier protein in *E. coli* only after the addition of an electron donor, D-lactate. They conclude that upon "energization" of the membrane the *lac* carrier protein undergoes conformational changes and then binds dansyl galactosides. The D-lactate or succinate dependent reduction of spin-labeled stearic acids in membrane vesicles may be analogous to the energy-dependent binding of dansyl galactosides to the *lac* carrier protein in *E. coli*. It should be pointed out that there is a difference between these two systems. Whereas the conformational changes observed by Schuldiner *et al.* require the electron flow throughout the electron transfer chain, the reduction of spin-labels does not require electron flow after cytochrome b_1.

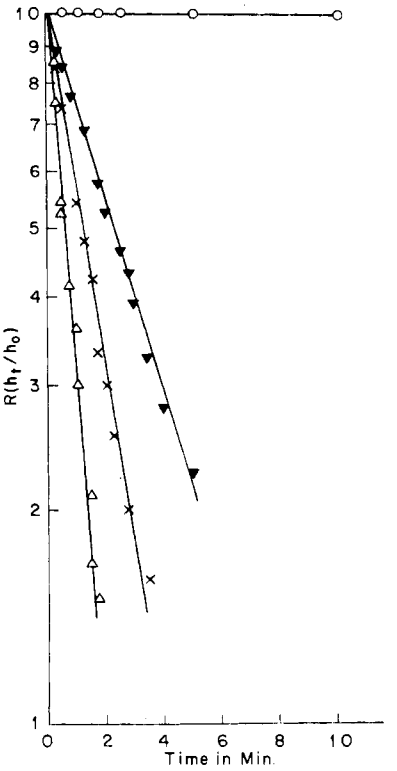

FIGURE 4: Rates of reduction of spin-labeled stearic acids I(12,3), I(5,10), and I(1,14). Membrane vesicles in 10 mM potassium phosphate (pH 6.6) and 10 mM magnesium sulfate were spin-labeled as described in the Experimental Section. Lithium D-lactate was added to a final concentration of 20 mM. The spectra were recorded at 30° and the epr signal intensity ratios (R) were calculated as described in the Experimental Section. (○) no addition of D-lactate (control); (△) 20 mM D-lactate in spin-label I(12,3); (x) 20 mM D-lactate in spin-label I(5,10); (▼) 20 mM D-lactate in spin-label I(1,14).

Present experimental results do not allow us to decide which one of the two schemes mentioned above is the correct mechanism for the D-lactate or succinate dependent reduction of spin-labeled stearic acids incorporated into *E. coli* membrane vesicles. By selecting appropriate mutants along the electron transfer chain as well as by using suitable spin-labels and inhibitors, spin-labeling technique can be used to investigate active transport in biological membranes.

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Cooperativity in Associating Proteins. Monomer–Dimer Equilibrium Coupled to Ligand Binding†

Alexander Levitzki* and Joseph Schlessinger

ABSTRACT: The cooperativity due to ligand binding to a protein monomer and a protein dimer in equilibrium was studied. The system is: E + E ⇌ E_2 (K_1); E + S ⇌ ES (K_2); ES + E ⇌ E_2S (K_3); and ES + ES ⇌ E_2S_2 (K_4). Using a computer and a plotter, Hill plots for differenct combinations of the parameters K_1, K_2, K_3, and K_4 were constructed. It was found that high ratios of K_4/K_3 with moderate K_1 and K_2 ensure high positive cooperativity. In such cases the species E_2, ES, and E_2S never accumulate to significant concentrations throughout the titration. High K_4/K_3 ratios or high K_4 alone does not ensure binding of ligand. The monomer must have a finite affinity toward the ligand in order for the whole binding process to occur. Ratios of K_4/K_3 lower than 10^3 generate negative cooperativity and the transient accumulation of E_2S in the protein–ligand mixture. The possible physiological significance of positive cooperativity due to protein aggregation is discussed.

Cooperativity in multisubunit enzymes is due to changes in the strength of subunit interactions coupled to ligand binding (Monod *et al.*, 1965; Koshland *et al.*, 1966). The modulation of subunit interactions by ligand binding brings about positive cooperativity, negative cooperativity, and mixed-type cooperativity (Levitzki and Koshland, 1969).

Some proteins dissociate upon ligand binding, and others associate in the presence of ligands (Levitzki and Koshland, 1972, and references therein; Duncan *et al.*, 1972). In such cases the ligand binding is always positively cooperative, as was already pointed out (Klotz *et al.*, 1970).

The analytical treatment of ligand binding coupled to association or dissociation is rather complex. We therefore decided to conduct a quantitative treatment, using the computer, of a case in which a monomer ⇌ dimer equilibrium is coupled to ligand binding.

Theory

Let us consider an enzyme E which binds the ligand S according to the following scheme

$$E + E \overset{K_1}{\rightleftharpoons} E_2 \quad (1)$$

$$E + S \overset{K_2}{\rightleftharpoons} ES \quad (2)$$

$$ES + E \overset{K_3}{\rightleftharpoons} E_2S \quad (3)$$

$$ES + ES \overset{K_4}{\rightleftharpoons} E_2S_2 \quad (4)$$

where K_1, K_2, K_3, and K_4 are the intrinsic association constants. One can write

$$[E_2] = K_1[E]^2 \quad (5)$$

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