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Functional molecular weight of the *lac* carrier protein from *Escherichia coli* as studied by radiation inactivation analysis

(target theory/proton electrochemical gradient/symport/oligomerization/reconstitution)

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ABSTRACT **Cytoplasmic membrane vesicles prepared from *Escherichia coli* containing multiple copies of the *lac y* gene were frozen in liquid nitrogen before or after generation of a proton electrochemical gradient (interior negative and alkaline) and irradiated with a high-energy electron beam at −135°C. Subsequently, the *lac* carrier protein was extracted into octyl β-D-glucopyranoside, reconstituted into proteoliposomes, and assayed for transport activity. Under all conditions tested, activity decreased as a single exponential function of radiation dosage, allowing straightforward application of target theory for determination of functional molecular mass. When *lac* carrier activity solubilized from nonenergized vesicles was assayed, the results obtained were consistent with a functional molecular size of 45–50 kDa, a value similar to the size of the protein as determined by other means. Similar values were obtained when the octyl β-D-glucopyranoside extract was irradiated, and the target size observed for D-lactate dehydrogenase was in good agreement with the molecular size of this enzyme. Strikingly, when the same procedures were carried out with vesicles that were energized with appropriate electron donors prior to freezing and irradiation, a functional molecular size of 85–100 kDa was obtained for the *lac* carrier with no change in the target size of D-lactate dehydrogenase. In contrast, when the vesicles were energized under conditions in which the proton electrochemical gradient was collapsed, the target mass of the *lac* carrier returned to 45–50 kDa. The results indicate that the functional mass of the *lac* carrier protein is no greater than a dimer and suggest that the proton electrochemical gradient may cause an alteration in subunit interactions.**

The *lac* carrier protein or *lac* permease is an intrinsic membrane protein in *Escherichia coli* that catalyzes the coupled translocation of substrate with protons in a symport reaction (see ref. 1 for a recent review). Recently, the protein was purified to homogeneity in a functional state and shown to be the product of the *lac y* gene (2, 3). Proteoliposomes reconstituted with this single polypeptide species catalyze all of the transport activities observed in right-side-out (RSO) membrane vesicles (2–6) with comparable turnover numbers and apparent K_ms (1, 7). In addition, monoclonal antibodies against purified *lac* carrier protein have been prepared and characterized (8). One of the antibodies is directed against an epitope that is accessible on the external surface of the membrane, and it inhibits transport without altering the ability of the carrier to bind substrate or catalyze exchange across the membrane (8, 9).

The product of the *lac y* gene is a 46.5-kDa polypeptide containing 417 amino acid residues of known sequence (10). A preliminary secondary-structure model has been proposed (11) based both on circular dichroic measurements indicating that the protein has an exceptionally high α-helical content and on the hydropathic profile of the protein along its primary sequence. The model postulates that the protein consists of 12–13 α-helical segments that traverse the bilayer in a zigzag fashion similar to that suggested for bacteriorhodopsin. Proteolysis experiments with RSO and inside-out (ISO) vesicles containing photoaffinity-labeled *lac* carrier protein demonstrate directly that the polypeptide spans the membrane and that the binding site is contained within a transmembrane segment of the protein (12). Finally, kinetic studies (13–15), experiments with group-specific reagents (16, 17), and the demonstration that certain *lac y* mutations are dominant (18) have led to the suggestion that oligomerization may be important for *lac* carrier function (see refs. 1 and 2).

To study oligomeric structure, we have used radiation inactivation analysis (19) to estimate the functional mass of the *lac* carrier protein under different conditions. A preliminary report on some of these experiments has been presented (20).

MATERIALS AND METHODS

Growth of Cells and Preparation of Membrane Vesicles. *E. coli* T206, which harbors a hybrid plasmid containing the *lac y* gene, was grown and induced as described (21). RSO vesicles were prepared by osmotic lysis (22, 23), and ISO vesicles were prepared by passage through a French pressure cell at 8000 psi (1 psi = 6.89 kPa) (24).

Preparation of Vesicles for Irradiation. Aliquots (600 μl) of a suspension containing 2–5 mg of membrane protein per ml in 50 mM KP_i (pH 6.6) were placed in 2.0-ml glass ampules, rapidly frozen in liquid nitrogen, and sealed with an oxygen flame. Vesicles were frozen after incubation in the presence of given electron donors as follows: aliquots (600 μl) of a suspension of RSO vesicles containing no more than 2 mg of membrane protein per ml in 50 mM KP_i (pH 6.6) were placed in glass ampules and equilibrated to 25°C under an atmosphere of pure oxygen. Ascorbate (potassium salt adjusted to pH 6.6) and phenazine methosulfate (PMS) or lithium D-lactate were then added to final concentrations of 20 mM and 0.1 mM or 20 mM, respectively. Incubation was continued at 25°C under oxygen for 1 min in the presence of reduced PMS or for 5 min in the presence of D-lactate, at which time the

Abbreviations: RSO, right-side-out; ISO, inside-out; PMS, phenazine methosulfate; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; Oct-Glc, octyl β-D-glucopyranoside; Dns^6-Gal, 6′-(*N*-dansyl) aminohexyl 1-thio-β-D-galactopyranoside; $\Delta\psi$, membrane potential; D-LDH, D-lactate dehydrogenase; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; Gal-S-Gal, β-D-galactopyranosyl 1-thio-β-D-galactopyranoside; $\Delta\bar{\mu}_{H^+}$, the proton electrochemical gradient; Mrad, millions of rads.

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samples were submerged in liquid nitrogen and then sealed. Where indicated, samples were incubated under identical conditions with carbonylcyanide *m*-chlorophenylhydrazone (CCCP) at a final concentration of 10 μM.

Partial Purification of *lac* Carrier Protein. Partially purified *lac* carrier protein was prepared as described (3). T206 membranes were sequentially extracted with 5 M urea and 6% sodium cholate (wt/vol), and *lac* carrier protein was then solubilized in 1.25% octyl β-D-glucopyranoside (Oct-Glc) in the presence of *E. coli* phospholipids at 3.75 mg/ml. Aliquots (350 μl) of Oct-Glc extract containing about 0.25 mg of protein per ml were placed in 2.0-ml glass ampules, rapidly frozen in liquid nitrogen, and sealed as described above.

Irradiation. Samples were irradiated as described (25) with a 13-MeV electron beam produced by a linear accelerator (Armed Forces Radiobiology Institute, Bethesda, MD). The beam was spread by a water scatterer to provide uniform exposure with <10% variation over a 225-cm^2 area at 90 cm from the beam port. Dose rate was typically 30 Mrad (millions of rads)/hr (1 rad = 10^{-2} gray). Thermoluminescent dosimeters were used before and after each series of radiations. Samples in sealed glass ampules were maintained at -135 ± 2°C during exposure by a stream of cold nitrogen gas, and temperature was monitored continuously with a platinum resistance probe (Yellow Springs Instrument). Before and after irradiation, samples were transported in dry ice and stored at −70°C or lower.

Reconstitution. The *lac* carrier protein was solubilized with Oct-Glc and reconstituted by detergent dilution as described (3–6, 26). The following protocol was typical: a frozen irradiated sample was thawed at 37°C immediately prior to use, and an aliquot (550 μl) was adjusted to pH 7.5 by adding 30 μl of 1.0 M dibasic KP_i, followed by 7 μl of 0.1 M dithiothreitol/26 μl of 560 mM lactose/56 μl of sonicated *E. coli* phospholipids (50 mg/ml)/61 μl of 15% Oct-Glc (wt/vol in 50 mM KP_i, pH 7.5). After a 10-min incubation at 0°C, the sample was centrifuged for 2 hr at 40,000 rpm in a Beckman type 50 rotor, and the supernatant was carefully aspirated. An aliquot (640 μl) of the supernatant was mixed with 192 μl of sonicated phospholipids and 17 μl of 15% Oct-Glc, agitated on a Vortex mixer, and incubated at 0°C for 20 min. The suspension was then squirted into 24 ml of 50 mM KP_i, pH 7.5/1 mM dithiothreitol, which was at room temperature. For counterflow experiments, the dilution solution was supplemented with 20 mM lactose. Proteoliposomes were then collected by centrifugation for 2 hr at 45,000 rpm in a Beckman type 60 rotor and resuspended in 260 μl of 50 mM KP_i, pH 7.5/1 mM dithiothreitol (for counterflow experiments, 20 mM lactose was also present) to a final protein concentration of about 0.6 mg/ml and a phospholipid concentration of 37.5 mg/ml. All samples were frozen and stored in liquid nitrogen. Immediately prior to transport assays, the samples were thawed at room temperature and sonicated for about 15 sec by using a bath-type sonicator as described (4–6).

Transport Assays. Transport in proteoliposomes reconstituted with solubilized *lac* carrier protein was assayed by using the following procedures: (*i*) [1-^{14}C]Lactose counterflow. Counterflow of internal lactose with external [1-^{14}C]lactose was carried out with proteoliposomes prepared in the presence of 20 mM lactose as described (3, 5). (*ii*) 6′-(*N*-Dansyl) aminohexyl 1-thio-β-D-galactopyranoside (Dns^6-Gal) counterflow. An aliquot (5 μl) of proteoliposomes prepared in the presence of 20 mM lactose was diluted into a cuvette containing 1.5 ml of 50 mM KP_i (pH 7.5) with 10 μM Dns^6-Gal. Fluorescence was recorded at 500 nm (excitation, 340 nm) using a Perkin–Elmer MPF-4 spectrophotofluorimeter (27). (*iii*) Membrane potential ($\Delta\psi$)-driven [1-^{14}C]lactose accumulation. $\Delta\psi$-driven lactose accumulation in proteoliposomes made in the absence of lactose was measured as described (6). A 200-fold potassium diffusion gradient ($K^+_{in} \rightarrow K^+_{out}$) in the presence of 20 μM valinomycin was used to generate $\Delta\psi$ (interior negative).

D-Lactate Dehydrogenase (D-LDH). D-LDH activity was assayed spectrophotometrically as described (28) by measuring the decoloration of dichlorophenolindophenol at 600 nm in a 1.0-ml reaction mixture containing 100 mM KP_i, pH 7.5/80 μg of membrane protein/0.25 mM PMS/30 μg of dichlorophenolindophenol/5 mM potassium cyanide/20 mM lithium D-lactate.

H^+-ATPase Activity. Release of $^{32}P_i$ from [^{32}P]ATP was measured in the absence and presence of dicyclohexylcarbodiimide (DCCD) at a final concentration of 100 μM as described (24). Reaction mixtures (final volume, 100 μl) contained (in final concentrations) 50 mM Tris·HCl, pH 7.8/2 mM magnesium sulfate/80 μg of membrane protein/4 mM [γ-^{32}P]ATP (0.4 mCi/mmol; 1 Ci = 37 GBq).

Protein. Protein was assayed as described (3) with bovine serum albumin as standard.

Target Analysis of Inactivation Data. The fraction of surviving activity was plotted logarithmically as a function of radiation dosage. Data for each set of experiments were fitted to a straight line by using least-squares analysis. Each point was weighted equally except for the unirradiated controls, which were weighted twice, as duplicate control samples were used in each set of experiments. The slope of the function is equal to $1/D_{37}$, where D_{37} is the dose of radiation required to decrease activity to 37% of the unirradiated control (19). D_{37} (in Mrad) is related to the target size (mass) of the functional unit by the following relationship: target size (in kDa) = $6.4 \times 10^5/D_{37}S_t$, in which S_t is a temperature factor experimentally determined to be 2.8 for irradiation at −135°C (29).

RESULTS

Radiation Inactivation of *lac* Carrier Function. Although data will not be presented, irradiation of RSO vesicles at low dosage causes a marked increase in passive permeability, which precludes direct measurements of transport activity after irradiation. For this reason, a protocol was used in which the *lac* carrier protein was solubilized from the membrane and then reconstituted into proteoliposomes (3–6, 26). Experiments were carried out with RSO and ISO vesicles, and duplicate samples were irradiated and reconstituted independently at each dosage. At least two types of transport activity were assayed after reconstitution (Fig. 1). As shown, when counterflow with [^{14}C]lactose (*A*) or Dns^6-Gal (*B*) and $\Delta\psi$-driven active transport (*C*) were examined, both the initial rates and maximum levels of accumulation decrease markedly with increasing dosage of radiation and eventually approach the levels observed when unirradiated carrier is assayed in the presence of high concentrations of β-D-galactopyranosyl 1-thio-β-D-galactopyranoside (Gal-S-Gal) (curves i).

When initial rates are plotted as a percentage of original activity versus radiation dosage, activity decays as a single exponential function of dosage, as determined with each type of assay (Fig. 2), thus allowing straightforward application of target theory for determination of functional molecular mass (19). The lines presented were determined from least-square fits of data from two to four transport assays with three sets of samples reconstituted independently, and the average results of the experiments are given in Table 1. The results yield a D_{37} value of 37 ± 1 Mrad, which corresponds to a radiation-sensitive mass of 49 kDa (see Table 1). This value is in good agreement with the molecular size of the *lac* carrier protein (46.5 kDa), as determined from the DNA sequence of the *lac y* gene (10) and from the amino acid composition of the purified polypeptide (3).

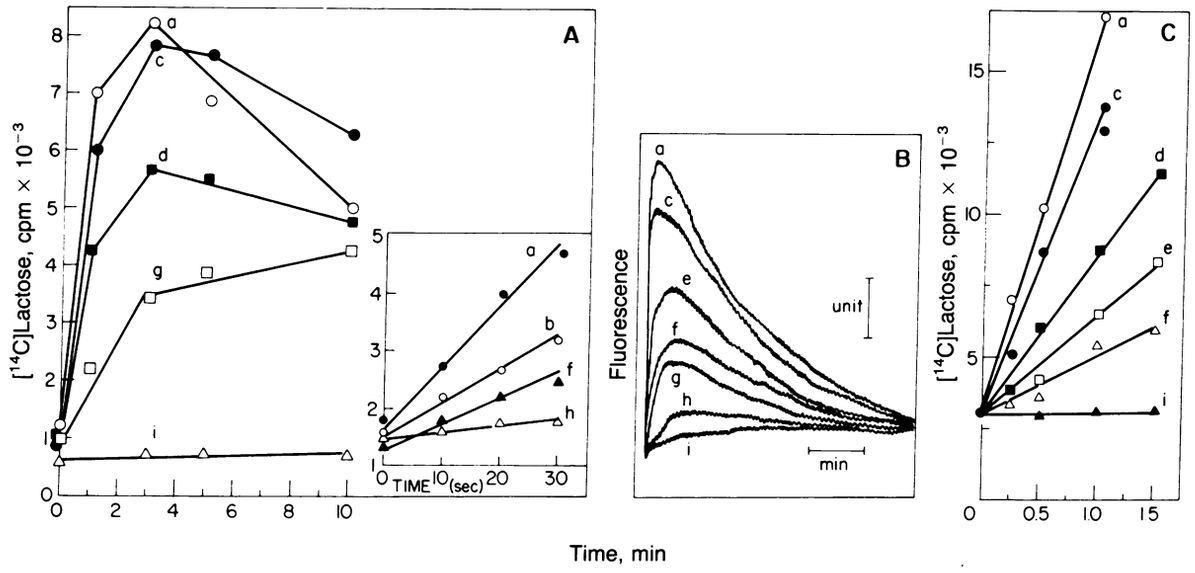

FIG. 1. Effect of radiation on [^{14}C]lactose counterflow (*A*), Dns^6-Gal counterflow (*B*), and $\Delta\psi$-driven [^{14}C]lactose accumulation (*C*). RSO vesicles from *E. coli* T206 were frozen, irradiated, extracted with Oct-Glc, reconstituted, and assayed for a given activity. Curves a–h were obtained with samples exposed to 0, 6, 12, 24, 36, 48, 60, and 72 Mrad, respectively. Curves i were obtained with unirradiated control samples assayed in the presence of 20 mM Gal-S-Gal, a potent competitive inhibitor. Initial rate data are highlighted in *A* (*Inset*) and in *C*. For clarity of presentation, only designated portions of the data are presented.

Experiments were also carried out in which the *lac* carrier protein was partially purified *in situ* by sequential extraction of T206 membranes with urea and cholate prior to solubilization in Oct-Glc, followed by irradiation and reconstitution. Again, a single exponential function is observed when remaining activity is plotted as a function of dosage (data not shown), and an average functional molecular size of 46 kDa is calculated from the D_{37} (see Table 1).

Effect of the Proton Electrochemical Gradient ($\Delta\bar{\mu}_{H^+}$). To investigate the effect of $\Delta\bar{\mu}_{H^+}$ on the target size of the *lac* carrier protein, RSO vesicles from *E. coli* T206 were incubated in the presence of reduced PMS or D-lactate under conditions that lead to the generation of a $\Delta\bar{\mu}_{H^+}$ (interior negative and alkaline) of about −170 mV (30) and then frozen quickly. Alternatively, samples were treated identically in the presence of 10 μM CCCP, a protonophore that collapses $\Delta\bar{\mu}_{H^+}$. Subsequently, the membranes were irradiated, and the *lac* carrier was solubilized, reconstituted, and assayed (Fig. 3).

Strikingly, *lac* carrier activity extracted from vesicles energized with reduced PMS (Fig. 3*A*) or D-lactate (Fig. 3*B*) prior to irradiation exhibit D_{37}s of 18 ± 2 and 21 ± 1 Mrad, respectively (solid lines), which correspond to functional molecular weights of 100 and 87 kDa (Table 1). In contrast, when identical incubations were carried out in the presence of CCCP, the D_{37}s increase to 39 ± 2 and 39 ± 1 Mrad, respectively (broken lines), reflecting an apparent decrease in target mass to 47 and 46 kDa (Table 1). The results imply that imposition of $\Delta\bar{\mu}_{H^+}$ across the membrane causes the functional molecular weight of the *lac* carrier protein to increase by a factor of 2.

D-LDH and the H^+-ATPase. As internal controls for the *lac* carrier protein, radiation inactivation was carried out with two other membrane-bound enzymes in *E. coli*. D-LDH is a single polypeptide species with a molecular size of ≈65 kDa that contains 1 mol of flavin adenine dinucleotide per mol of enzyme (28). Target analysis of D-LDH activity in T206 vesicles yields a functional molecular size of 58 kDa [D_{37} = 31 ± 2 Mrad], and no significant difference is observed when the vesicles are energized under O_2 prior to freezing and irradiation (Fig. 4, compare open and closed circles). The H^+-ATPase, on the other hand, is an oligomer containing multiple nonidentical subunits, and molecular sizes of 382 and 529 kDa have been estimated for the F_1 portion and for the intact F_1F_0 complex, respectively (31). Radiation inactivation analysis of DCCD-sensitive ATPase activity, which is presumably a measure of the intact F_1F_0 complex, exhibits a functional molecular size of 320 kDa [D_{37} = 5.6 ± 0.6 Mrad; Fig. 4). The value is significantly lower than predicted; however, it is readily apparent that the target mass of this enzyme complex is considerably greater than that of D-LDH or the *lac* carrier protein.

DISCUSSION

Although evidence bearing on the subunit structure of the *lac* carrier protein is sparse, it has been suggested that oligomerization may play an important role in *lac* carrier function

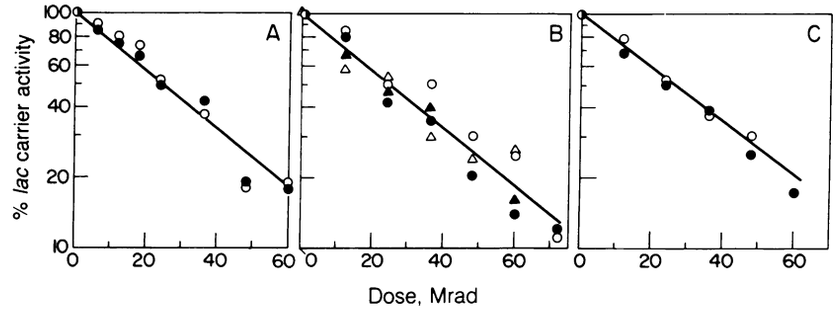

FIG. 2. Decay curves for radiation inactivation of *lac* carrier activity. Experiments similar to those shown in Fig. 1 were carried out with RSO (○, △) and ISO (●, ▲) T206 vesicles. Fractional initial rates (initial rate of irradiated sample per initial rate of control) of [^{14}C]lactose counterflow (*A*), Dns^6-Gal counterflow (*B*), and $\Delta\psi$-driven [^{14}C]lactose uptake (*C*) are plotted as a function of radiation dosage. See also Table 1.

Table 1. Compilation of radiation inactivation data for the *lac* carrier protein

Preparation	Assay	No. of exp.	D_{37}*, Mrad	Size†, kDa	Mean size‡, kDa
ISO or RSO vesicles	Dns^6-Gal counterflow	4	36 ± 3	50	49 ± 1
	Lactose counterflow	2	37 ± 1	48	
	ΔΨ-driven transport	2	37 ± 2	48	
Oct-Glc extract	Dns^6-Gal counterflow	1	34	53	46 ± 8
	Lactose counterflow	2	47 ± 21	38	
RSO vesicles					
With ascorbate/PMS	Lactose counterflow	5	18 ± 1	100	100
	ΔΨ-driven transport	2	18 ± 2	100	
With ascorbate/PMS and CCCP	Lactose counterflow	2	38 ± 1	47	47 ± 0.3
	ΔΨ-driven transport	2	39 ± 3	46	
With D-lactate	Dns^6-Gal counterflow	1	20	90	87 ± 5
	Lactose counterflow	2	20 ± 1	90	
	ΔΨ-driven transport	1	23	78	
With D-lactate and CCCP	Dns^6-Gal counterflow	1	40	45	46 ± 0.3
	Lactose counterflow	1	39	46	
	ΔΨ-driven transport	1	39	46	

*The D_{37}s given are mean values for each type of experiment ± the average deviation where appropriate.
†Calculated from the mean values of D_{37}.
‡Mean molecular mass ± the average deviation. The number of experiments carried out was weighted in the calculations.

(1, 2). Kinetic studies show that $\Delta\bar{\mu}_{H^+}$ causes a marked decrease in apparent K_m, and quantitative measurements under various conditions show that the distribution of the transport reaction between the high and low apparent K_m pathways varies as the square of $\Delta\bar{\mu}_{H^+}$ (13–15). Inactivation of lactose transport by various maleimides (17) and by the histidine reagent diethylpyrocarbonate (16) is enhanced 2- to 3-fold in the presence of $\Delta\bar{\mu}_{H^+}$, suggesting that the shift between the two kinetic pathways may involve structural changes that alter the reactivity of certain functional groups in the *lac* carrier protein. Recent genetic studies indicate that certain *lac y* mutations are dominant (18). Based primarily on the kinetic evidence, it was tentatively suggested (1, 2, 14) that the *lac* carrier might exist in two forms, monomer

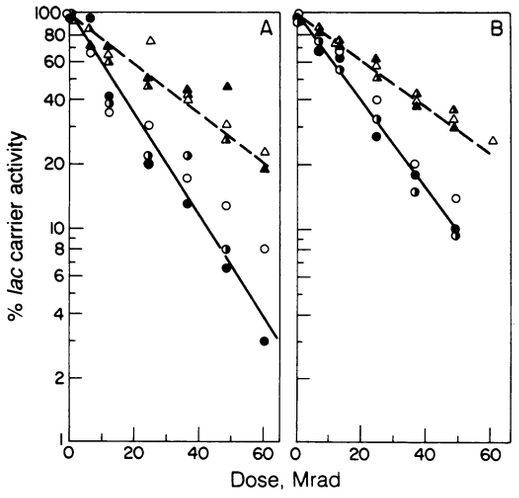

FIG. 3. Effect of $\Delta\bar{\mu}_{H^+}$ on radiation inactivation of *lac* carrier activity. RSO vesicles prepared from *E. coli* T206 were incubated in the presence of reduced PMS (*A*) or D-lactate (*B*) prior to freezing and irradiation (solid lines). Where indicated (broken lines), the samples also contained 10 μM CCCP. Fractional initial rates (initial rate of irradiated sample per initial rate of control) of [^{14}C]lactose counterflow, Dns^6-Gal counterflow, and Δψ-driven [^{14}C]lactose uptake are plotted as a function of radiation dosage. (*A*) ◑, ●, ◬, ▲, [^{14}C]lactose counterflow; ○, △, Δψ-driven [^{14}C]lactose uptake. (*B*) ◑, ◬, [^{14}C]lactose counterflow; ●, ▲, Dns^6-Gal counterflow; ○, △, Δψ-driven lactose uptake. See also Table 1.

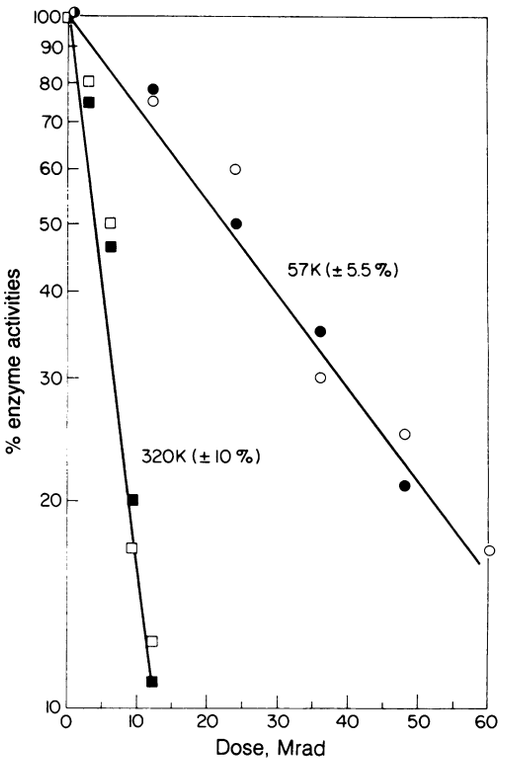

FIG. 4. Radiation inactivation of D-LDH and H$^+$-ATPase activities in T206 vesicles. Vesicles were frozen, irradiated, and assayed for a given activity. ○ and ●, D-LDH activity in vesicles frozen and irradiated after incubation in the absence and presence of 20 mM D-lactate, respectively; □ and ■, DCCD-sensitive ATPase activity in RSO and ISO vesicles, respectively. Fractional activities (initial rate of irradiated sample per initial rate of control) are plotted as a function of radiation dosage.

and dimer, that the monomer catalyzes translocation through a high K_m pathway (facilitated diffusion), and the dimer through a low K_m pathway (active transport), and finally, that $\Delta\bar{\mu}_{H^+}$ causes a monomer–dimer transition.

The results presented here indicate that if the *lac* carrier is an oligomer, it is probably not larger than a dimer. This conclusion seems reasonable in view of the finding that under no conditions tested does radiation inactivation yield a target mass greater than twice the molecular weight of the monomer. Remarkably, moreover, the studies also support the notion that $\Delta\bar{\mu}_{H^+}$ may alter interactions between subunits of the putative dimer. Thus, *lac* carrier activity solubilized from nonenergized vesicles exhibits a functional molecular mass of 45–50 kDa, a value similar to that determined by other means (3, 10), and a similar target size is obtained on irradiation of Oct-Glc extracts containing the protein. When the same procedure is carried out with vesicles energized prior to freezing and irradiation, however, a functional molecular mass of 85–100 kDa is observed for the *lac* carrier protein with no significant change in the target mass of D-LDH. Finally, when CCCP is added during energization to collapse $\Delta\bar{\mu}_{H^+}$, the functional molecular mass of the *lac* carrier protein returns to 45–50 kDa.

One aspect of radiation inactivation analysis that makes the technique particularly attractive for membrane proteins is its potential to yield structural information from functional studies carried out *in situ* (19). The recent findings of Schlegel *et al.* (25), for instance, show that activators of adenylate cyclase alter its functional molecular mass in a manner suggesting that receptor occupancy triggers cyclase activation by inducing oligomerization. Based on target analysis of cytochalasin B binding (32) and D-glucose fluxes (33), it has been postulated that the D-glucose carrier in the human erythrocyte membrane is an oligomer. Inactivation studies on purified H^+/K^+-ATPase (34) indicate that this pump may function as a trimer, and similar studies with purified and native D-β-hydroxybutyrate dehydrogenase have led to the proposal that this enzyme is a tetramer (35).

The radiation target sizes of more than 30 different enzymes correspond either to the entire molecule or to the subunit mass (19). In the latter case, radiation damage to one subunit clearly does not result in loss of activity of other subunits. However, when enzymatic activity is lost after a single ionization among several subunits (i.e., when the target size is the entire molecule), at least two explanations are possible. First, the enzymatic function could require the simultaneous presence of two or more peptide chains—for conformational stability perhaps, or because the catalytic mechanism may involve the structure of more than one subunit. A second possibility (19) is that radiation damage to one subunit might cause structural disruption in other subunits by energy transfer with resultant covalent bond breakage. The second possibility has been examined recently (36) with a single enzyme, glutamate dehydrogenase, which exhibits a hexamer size as the radiation-sensitive unit for enzymatic activity. Subunit structure is destroyed only when a primary ionization occurs within that polymer, and no evidence was found for massive energy transfer between subunits.

In the present study, the *lac* carrier protein in nonenergized membranes exhibits the functional molecular weight of a monomer; twice this mass is observed in the presence of $\Delta\bar{\mu}_{H^+}$. Because purified *lac* carrier protein catalyzes all of the transport reactions observed in the native membrane (1, 7), it is most likely that the larger target size does not involve other protein species. Thus, two possibilities seem apparent. (*i*) In the nonenergized membrane, the *lac* carrier is a monomer, and dimerization occurs in the presence of $\Delta\bar{\mu}_{H^+}$. (*ii*) Membranous *lac* carrier is always a dimer, and generation of $\Delta\bar{\mu}_{H^+}$ causes an increased interaction between subunits.

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