Bacterial Phosphoenolpyruvate-Dependent Phosphotransferase System: Association State of Membrane-Bound Mannitol-Specific Enzyme II Demonstrated by Radiation Inactivation†

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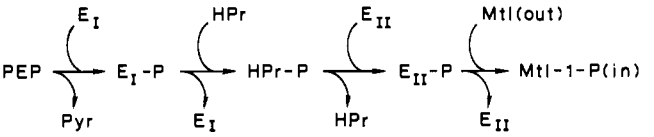
ABSTRACT: The quaternary structure of the membrane-bound mannitol permease (E_{II}^{Mtl}) of the bacterial phosphotransferase system in *Escherichia coli* has been investigated in the membrane by using the radiation inactivation method. The experiments reveal two distinct but interconvertible forms of the permease. The first state is a dimer, and the second state consists of a less active higher molecular weight complex involving the dimer. The equilibrium between these two forms in the membrane can be shifted by changing the pH. At pH 8.1 the dimer is the dominant form. Decreasing the pH results in increased binding of a regulatory protein to the dimer, thus increasing the amount of the higher molecular weight form involving the dimer. Cross-linking E_{II}^{Mtl} in situ, followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting, resulted in the formation of two cross-linked forms. One is the dimer, and the other has a higher molecular weight. Two-dimensional electrophoresis using a reversible cross-linker revealed no other protein except E_{II}^{Mtl} in these complexes.

The bacterial phosphoenolpyruvate sugar phosphotransferase system (PTS)[1] catalyzes the concomitant transport and uptake of many sugars [for review see Postma and Lengeler (1985)] by a mechanism similar to the one shown in Scheme I for mannitol. The general PTS proteins, E_I and HPr, are soluble; the sugar-specific permease, E_{II}^{Mtl}, is an integral membrane protein. The latter is directly responsible for phosphorylation and transport of mannitol. A second reaction also catalyzed by E_{II}^{Mtl} is the exchange reaction in Scheme II (Saier et al., 1977).

Purified E_{II}^{Mtl} consists of a single polypeptide chain with a molecular mass of 60 (±5%) kDa determined by SDS–polyacrylamide gel electrophoresis (Jacobson et al., 1979) and 68 kDa determined from the gene sequence (Lee & Saier, 1983). The mechanism by which E_{II} catalyzes the reactions in Schemes I and II has been the subject of several studies. The results of some of these studies suggested that association of the protein was necessary for expression of its enzymatic activity. Kinetics of the exchange reaction catalyzed by the purified enzyme indicated that the functional form of the enzyme was an oligomer (Saier, 1980; Roossien et al., 1984). Roossien et al. (1986) showed that the purified enzyme could be cross-linked to a dimer by thiol-specific cross-linkers as well as oxidizing reagents. Dimers of E_{II}^{Mtl} and P–E_{II}^{Mtl} have also been extracted from labeled membranes (Roossien & Robillard, 1984; Stephan & Jacobson, 1986a). These data are indicative of possible E_{II} structures, but they cannot be taken as proof of the structural state of the membrane-bound enzyme. All results so far have been obtained with detergent-treated or purified preparations. Detergent treatment could mask the occurrence of higher association states in the membrane. The present report examines the association state of membrane-bound E_{II}^{Mtl} by using radiation inactivation analysis.

Scheme I



Scheme II

$$*\text{Mtl} + \text{Mtl-1-P} \xrightleftharpoons{E_{II}} *\text{Mtl-1-P} + \text{Mtl}$$

This technique for determing molecular mass is based on the fact that the probability of destroying a protein molecule by high-energy radiation is a function of its molecular volume. The technique can be used on crude preparations, thus enabling the determination of the molecular mass in situ.

Our experiments show that the dimer is the minimum structural unit of E_{II} in the membrane. A higher molecular weight complex with reduced activity occurs at lower pH.

Materials and Methods

D-[1-^{14}C]Mannitol (59 mCi/mmol) was obtained from Amersham. Lubrol PX, dimethyl 3,3′-dithiobis(propionimidate), hexylagarose, butylagarose, sodium deoxycholate, the potassium salt of phosphoenolpyruvate, and the barium salt of mannitol 1-phosphate were from Sigma. Sodium deoxycholate was recrystallized twice from acetone/H_2O. The sodium salt of mannitol 1-phosphate was prepared from the

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[1] Abbrevations: PTS, phosphoenolpyruvate-dependent phosphotransferase system; PEP, phosphoenolpyruvate; Mtl, mannitol; Mtl-1-P, mannitol 1-phosphate; Pyr, pyruvate; α-MG, methyl α-glucopyranoside; E_I, enzyme I; HPr, histidine-containing phosphocarrier protein; E_{II}^{Mtl}, mannitol-specific enzyme II; E_{II}^{Glc}, glucose-specific enzyme II; ADH, alcohol dehydrogenase; D-LDH, D-lactate dehydrogenase; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; PMSF, phenylmethanesulfonyl fluoride; GARPO, goat anti-rabbit IgG (H + L)–horseradish peroxidase conjugate; HRP, horseradish peroxidase; KP_i, potassium phosphate; NaP_i, sodium phosphate; MES, 2-(*N*-morpholino)ethanesulfonic acid; BSA, bovine serum albumin; NAD, nicotinamide adenine dinucleotide.

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barium salt as described by Roossien et al. (1984). Goat anti-rabbit IgG (H + L)–horseradish peroxidase conjugate and HRP color development reagent were from Bio-Rad. E_I and HPr were purified from *Escherichia coli* P-650 as described previously (Dooijewaard et al., 1979; Robillard et al., 1979). Inside-out vesicles were prepared from *E. coli* ML 308-225 cells grown on mannitol. Frozen cells were thawed and suspended in 20 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM sodium azide, and 1 mM PMSF (1 g of cell pellet/10 mL) and passed through a French press at 8000 psi. The suspension was centrifuged at 30000*g* for 10 min. The supernatant was then centrifuged at 200000*g* for 45 min. The supernatant was discarded, and the pellet was resuspended in the same buffer as above but at pH 8.4, by use of a glass Potter homogenizer, and recentrifuged. The pellet was suspended in the pH 8.4 buffer (1 mL per gram of starting cell wet weight) and stored in small portions in liquid N_2 until used.

E_{II}^{Mtl} Assays. The phosphorylaton and transphosphorylation activities were measured by following the amount of [^{14}C]-Mtl-P formed. The sugar phosphate was separated from nonphosphorylated sugar by the Dowex AG1-X2 ion-exchange column procedure (Misset et al., 1980) with 0.2 N HCl in place of LiCl as the eluent. Specific conditions for each experiment can be found in the figure legends.

Yeast ADH and E. coli D-*LDH Activity Measurements.* ADH was dissolved in 1% BSA containing 10 mM DDT and irradiated simultaneously with the vesicles but in separate capillary tubes. After irradiation, 60 μL was mixed with 10 μL of 0.1% BSA containing 10 mM DDT, and these stock samples were kept in liquid nitrogen until used. At the time of assaying, the stock sample was diluted 40 times in 0.1% BSA and 1 mM DTT. One hundred microliters was added to 2.9 mL of 65 mM pyrophosphate buffer, pH 9.2, containing 65 mM glycine, 19 mM semicarbazide hydrochloride, 18 mM NAD, and 3.3% ethanol, and the ADH activity was recorded at 340 nm.

D-LDH activity was assayed on the same vesicles as E_{II}^{Mtl} activity by monitoring the reduction of an acceptor dye, dichlorophenolindophenol (DCPIP), at 600 nm in the presence of phenazine methosulfate (PMS) (Kaczorowksi et al., 1978). Since the DTT, used as a scavenger during irradiation, interfered strongly with the assay system, the activity was measured by use of identical samples with and without D-lactate. Vesicles (10–20 μL) were mixed with 3.9 mL of 100 mM KP_i, pH 7.5, 5 mM KCN, 0.4 mM PMS, and 1.1 mM DCPIP. The mixture was divided. An 1800-μL aliquot was mixed with 100 μL of 100 mM lithium D-lactate, and 1800 μL was diluted with 100 μL of H_2O. The change in absorbance of the two samples at 600 nm was monitored simultaneously in a Cary double-beam spectrophotometer.

Irradiation and Calibration Procedure. Seventy-five-microliter vesicle aliquots, 5.15 mg of membrane protein/mL, suspended in 20 mM Tris-HCl, pH 8.4, and 5–10 mM DTT, were put in glass capillaries and frozen in liquid nitrogen. Samples were irradiated by using two different systems. The first system employed 16-MeV electrons from a Philips MEL SL 75/20 20-MeV linear accelerator at the Department of Radiotherapeutics, New Addenbrooks Hospital, Cambridge, England. The dose rate was 2 Mrad/min. During irradiation the vesicles were kept in liquid nitrogen. The machine was routinely calibrated by Perspex dosimetry. Yeast alcohol dehydrogenase (molecular mass 150 kDa) irradiated simultaneously and D-lactate dehdyrogenase [molecular mass 65 kDa, Campbell (1984)] present in the vesicles [for target volumes see Lo et al. (1982) and Goldkorn et al. (1984)] served as controls to establish the relationship between dosage and molecular mass under our specific irradiation conditions.

The second system employed was a ^{60}Co source at the Interuniversity Reactor Institute at Delft, Holland. The dose rate was 0.41 Mrad/h. The samples were kept at −50 °C by blowing cold nitrogen gas through the irradiation chamber. After irradiation the vesicles were kept in liquid nitrogen until use.

Data were treated according to the classical target theory (Kempner & Schlegel, 1979). The percentage of surviving activity was plotted logarithmically versus the dose. If a straight curve was obtained, the slope was determined by linear regression and the molecular mass calculated by comparison to the biological controls.

Antibody Preparation. Antibodies to E_{II}^{Mtl} were raised in rabbits by three subsequent injections of 300 μL of purified E_{II}^{Mtl} mixed with an equal volume of Freund's complete adjuvant. For the third injection this was replaced by Freund's incomplete. Eleven days after the third injection the blood was collected, mixed with 0.01% NaN_3, and allowed to clot overnight. After a low-spin centrifuge step the supernatant was stored at −20 °C. The antiserum obtained was capable of inhibiting PEP-dependent phosphorylation of Mtl and reacting with purified E_{II}^{Mtl} in an immunoblot.

Immunoblotting Procedure. Gels were transferred electrophoretically to nitrocellulose by using the LKB 2005 Transphor electroblotting unit. The transfer buffer consisted of 200 mM glycine, 25 mM Tris, and 20% methanol. A current ranging from 0.4 to 1 A was applied for 90 min. The nitrocellulose sheet was then washed 8 times (10 min per wash) with buffer consisting of 0.5 M NaCl, 20 mM Tris-HCl, pH 7.5, and 0.05% Tween 20 and incubated with 0.1% antiserum in the buffer containing Tween overnight at room temperature on a rotator. After eight 10-min washes with the Tween buffer, the sheet was incubated with a 1:3000 solution of GARPO in the same buffer for 60 min. After six washes with the Tween buffer and two washes with buffer lacking Tween, the sheet was developed with 60 mg of HRP in 20 mL of ice-cold methanol mixed with 100 mL of the Tris/NaCl buffer containing 0.018% H_2O_2.

Purification of Cross-Linked E_{II}^{Mtl}. ML 308-225 cells (14 g) grown on mannitol were suspended in 80 mL of 50 mM KP_i, pH 7.5, and 1 mM PMSF. After passage through a French press (10000 psi) the cell debris was removed by a low-speed centrifugation. The vesicles were collected by a high-speed centrifugation, washed once with 40 mL of buffer, and suspended in 14 mL of the above buffer. DTT was absent in all steps.

Cross-linking was performed by mixing 1.8 mL of vesicles with 6.3 mL of 50 mM NaP_i, pH 8, and 90 μL of 90 mM dimethyl 3,3′-dithiobis(propionimidate). After 35 min the reaction was stopped with 2.16 mL of 100 mM NH_4 OAc. After 5 min 150 μL of non-cross-linked vesicles was added to monitor activity during purification. The reaction mixture was diluted in 20 mM Tris-HCl, pH 8.4, to a final volume of 45 mL containing 0.2 M NaCl and 0.5% deoxycholate (DOC). All insoluble material was removed by high-speed centrifugation. The supernatant was loaded on a 10.5-mL hexyl-agarose column and eluted with a gradient of 0–2% lubrol in 20 mM Tris-HCl, pH 8.4, containing 0.5% DOC and 0.2 M NaCl. E_{II}^{Mtl}-containing fractions (detected by immunoblotting) were pooled, extensively dialyzed against 20 mM Tris-HCl, pH 8.3, and 0.5 mM NaN_3, containing 0.5% lubrol, and diluted to 0.1% lubrol in the same buffer. After this sample was loaded on a 4-mL butylagarose column, E_{II}^{Mtl} was eluted

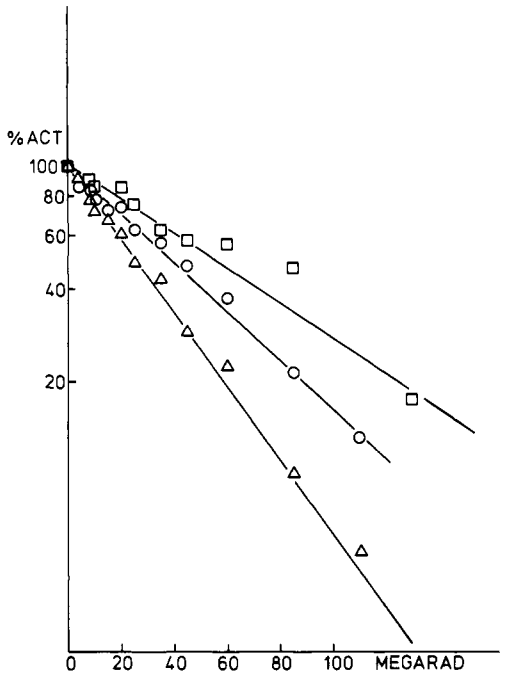

FIGURE 1: Radiation inactivation curves of (○) membrane-bound E_{II}^{Mtl} and calibration enzymes (△) yeast ADH and (□) *E. coli* D-LDH. ADH and LDH activity measurement procedures are described under Materials and Methods. E_{II}^{Mtl} activity was measured in the absence of detergent as indicated in the legend to Figure 4.

with a gradient of 0–300 mM NaCl in the above 0.1% lubrol containing buffer. E_{II}^{Mtl} fractions were separately frozen in liquid nitrogen until used.

Two-Dimensional (2D) Electrophoresis. Membranes were incubated for 30 min at 37 °C in 62.5 mM Tris-HCl, pH 8.8, 2% SDS, and 10% glycerol. In each experiment two parallel gels were run. The first dimension was run on a 1.3 mm thick 7.5% polyacrylamide–SDS slab gel according to Laemmli (1970). The second running gel was a 3 mm thick 7.5% polyacrylamide–SDS slab gel, but the stacking gel was a 1% agarose gel containing 50 mM DTT in the Laemmli stacking buffer. The strip from the first dimension gel was embedded in the agarose gel horizontal to the running direction. After electrophoresis, one gel was stained with silver (Wray et al., 1981) and the parallel gel was subjected to immunoblotting as described above.

RESULTS

Effect of Irradiation on PEP-Dependent Phosphorylation. Figure 1 presents radiation inactivation plots showing the loss of activity as a function of radiation dose for membrane-bound E_{II}^{Mtl} (○), D-lactate dehydrogenase in the same membrane preparation (□), and yeast alcohol dehydrogenase (△). The molecular mass of E_{II}^{Mtl} is intermediate between that of D-LDH, 65 000 daltons on the basis of the nucleic acid derived primary sequence (Cambell, 1984), and yeast ADH, 150 000 daltons. The estimated molecular mass of E_{II}^{Mtl} is 100 kDa. This value is intermediate between the E_{II}^{Mtl} monomer and dimer, 68 and 136 kDa, respectively, derived from the amino acid sequence (Lee & Saier, 1983). This point will be treated in detail after the following section.

The pH dependence of the activity remaining after irradiation is presented in Figure 2. The membranes used in all of the measurements were irradiated at one pH, 8.4. They were then diluted 20 times in 25 mM KP_i, pH 7.5, containing 1 mM DTT, and 10-μL samples were then used for activity measurements. An apparent linear decrease of activity resulted when the pH of the assay medium was 8.1 [Figure 2A (▽)].

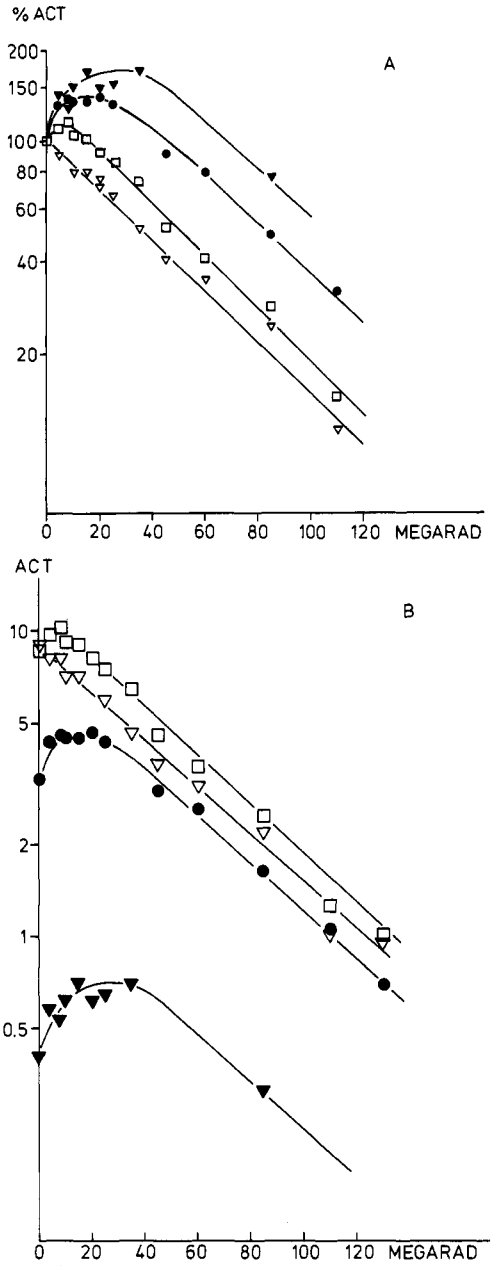

FIGURE 2: PEP-dependent phosphorylation measured as a function of radiation dose and pH. Assays (100 μL) were performed at 30 °C in the presence of 3–7 μL of membrane protein, 0.1 μM E_I, 12.4 μM HPr, 10 mM NaF, 6 mM $MgCl_2$, 3 mM DTT, 10 mM K-PEP, 1 mM [^{14}C]Mtl, and 50 mM buffer [(▼) 25 mM KP_i + 25 mM MES, pH 5.7; (●) 25 mM KP_i + 25 mM Tris-HCl, pH 6.7; (□) 25 mM KP_i + 25 mM Tris-HCl, pH 7.4; (▽) 25 mM KP_i + 25 mM Tris-HCl, pH 8.1]. The curves are normalized in panel A to the activity at zero radiation dose while in panel B absolute values are plotted. Unirradiated control values [μM [^{14}C]Mtl-1-P formed/(μg of membrane protein·min)] were as follows: pH 5.7, 0.4; pH 6.7, 3.3; pH 7.4, 8.7; and pH 8.1, 8.9.

The same batch of irradiated diluted vesicles assayed at pH 7.4 did not show a linear dose dependence. At low doses of irradiation an increase in activity was seen in comparison with the nonirradiated control activity also measured at pH 7.4 (□). This increase was followed by a decrease at higher doses. The effect was enhanced when the pH of the assay medium was decreased further to 6.7 (●) and 5.7 (▼). Both the maximum stimulation and the dose at which the maximum occurred increased as the pH decreased. The data in Figure 2B are replotted from Figure 2A as the absolute value of the activity at each pH versus the dose. They show that the absolute value decreases as the pH is lowered. The decrease in the absolute

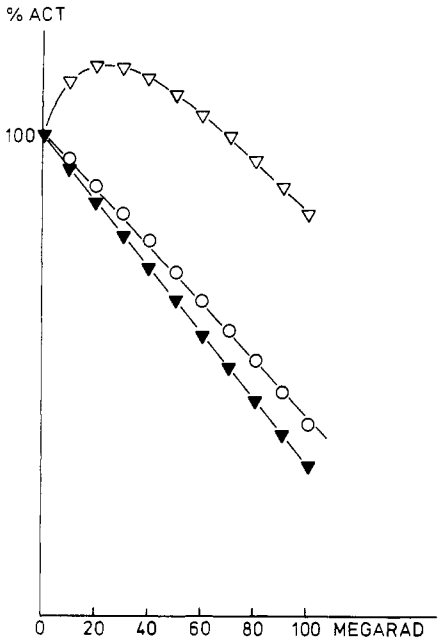

FIGURE 3: Computer simulation using a model in which the effect of inhibitor binding upon radiation inactivation analysis is shown. In this simulation the enzyme is twice the size of the inhibitor, and upon binding of the inhibitor the activity decreases by a factor of 9. (▼) No binding; (○) 40% binding; (▽) 100% binding. The curves are normalized to unit activity at zero radiation dose.

value of the activity was expected in light of the pH profile of native E_{II}^{Mtl}. The increase in activity after low doses of radiation, however, was unexpected. The simplest explanation of the increase in activity would be to assume that E_{II}^{Mtl} is subject to regulation by another protein. At low pH this protein would couple to E_{II} and diminish activity. The binding would be reversible and pH dependent:

$$R + E_{II} \rightleftarrows RE_{II}$$

According to the target theory, the activities surviving after irradiation of the uncoupled and coupled E_{II} are given by eq 1 and 2, respectively. μ_E and μ_R are constants reflecting the

$$A_D/A_0 = e^{-\mu_E D} \quad (1)$$

$$A_D/A_0 = (1/S)[Se^{-\mu_E D-\mu_R D} + (1 - e^{-\mu_R D})e^{-\mu_E D}] \quad (2)$$

molecular volumes of E_{II} and the regulator, respectively and S is the factor describing the relative activity of the enzyme–regulator complex compared to the free enzyme. The activity remaining after irradiation will be a sum of (1) and (2), the relative contribution of each term being fixed by the equilibrium and thus by the pH. A system consisting of a hypothetical enzyme with a regulator half its size has been simulated in Figure 3 by using these equations. The activity is assumed to decrease by a factor of 9 when the regulator is coupled. The sizes of the enzyme, regulator, and stimulation factor chosen are arbitrary and are not meant as values for the E_{II}^{Mtl} complex. When the regulator size and stimulation factor are both changed within certain limits, similar curves can be obtained. The model shows that 100% coupling [Figure 3 (▽)] results in a curve similar to that observed in Figure 2. Curves like this have been reported by Kincaid et al. (1981) for the calmodulin-activated cyclic nucleotide phosphodiesterase from brain and by Harmon et al. (1980, 1983) for the insulin receptor.

The high-pH activity measurments (▽) in Figure 2 decreased linearly with dose; no initial increase was observed at low doses. Coupling, however, could still be occurring, even if no initial increase occurred. This is shown in Figure 3 (○)

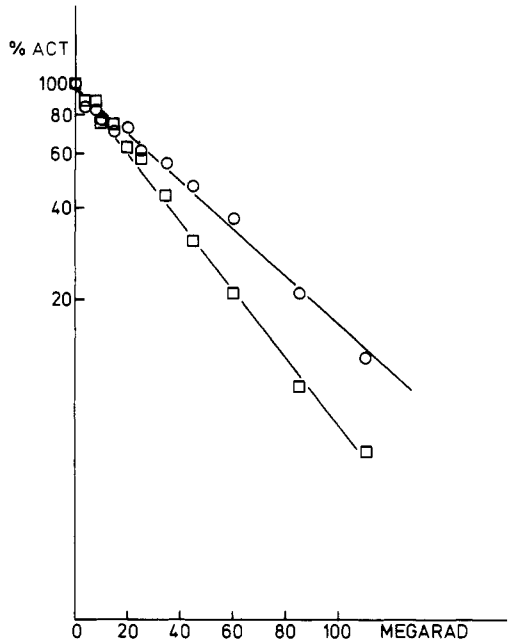

FIGURE 4: Influence of detergent on target volume. PEP-dependent phosphorylation was measured on the same vesicles used in Figure 2, in the presence of 0.1 μM E_I, 12.4 μM HPr, 10 mM NaF, 6 mM $MgCl_2$, 3 mM DTT, 10 mM K-PEP, 1 mM [^{14}C]Mtl, and 50 mM KP_i, pH 8.1. Before assaying, 10-μL vesicles were diluted with 10 μL of buffer containing no (○) or 1% lubrol (□). After 20 min at 30 °C the vesicles were diluted 10 times with buffer, and 10 μL was used for assaying. The end concentration of lubrol in the lubrol-containing assay was 0.01%. Curves were normalized to unit activity at zero radiation dose.

where only 40% of the hypothetical enzyme is in the complexed state. There is no initial increase at low dosage, but the slope of the curve is different from that of the uncomplexed enzyme, resulting in an underestimate of the target volume. In this particular example, the volume derived from the slope is 0.82 times the real volume. Following the same line of reasoning, the molecular mass of E_{II}, 100 kDa, derived from the pH 8.1 measurements in Figures 1 (○) and 2 (▽) could indicate that E_{II} is still partially complexed at this pH.

Change of Apparent Target Volume for PEP-Dependent Phosphorylation after Detergent Treatment. Previous experiments have shown that E_{II} dimers can be extracted, intact, from the membrane by mild detergent treatment (Roossien & Robillard, 1984; Lolkema et al., 1985; Stephan & Jacobson, 1986a). Figures 4 and 5 consider the influence of detergent on the form of the radiation inactivation curves. The same vesicles used in the above experiments were incubated in 1% lubrol for 20 min at 30 °C and then diluted to the same concentration as before. Again the PEP-dependent phosphorylation was measured at pH 8.1. The final lubrol concentration in the assay mixture was 0.01%. Plotting the activity in the same way as in Figure 2 again resulted in a curve decreasing as a straight line [Figure 4 (□)]. However, the slope of the line was steeper than that for non-detergent-treated enzyme (○) and yielded a target volume of 140 000 ± 20 000. The activity of the unirradiated control also increased (106–136%). The two target volumes obtained with the same irradiated vesicles with and without detergent imply that the detergent must, in some way, influence the system. These data, taken together with the pH effect of Figure 2, can be explained in terms of a pH-dependent association between an E_{II}^{Mtl} dimer and some macromolecule that reduces the phosphorylating activity of the dimer. The complex is strongly associated at low pH. Radiation-induced destruction of this macromolecule

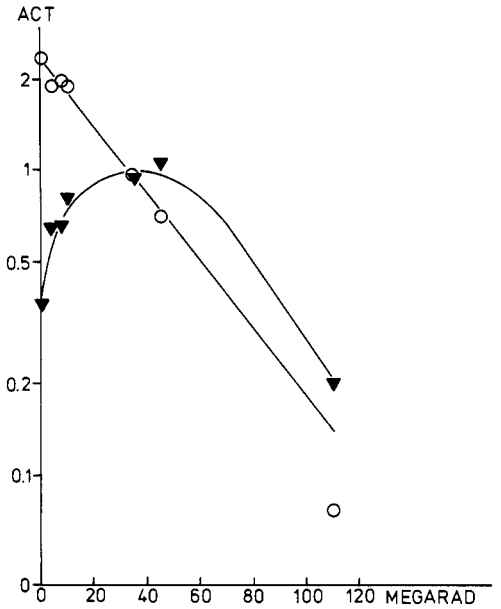

FIGURE 5: PEP-dependent phosphorylation measured as a function of radiation dose. Assays (100 μL) were performed at 30 °C in the presence of 3–7 μg of membrane protein, 0.1 μM E_I, 12.4 μM HPr, 10 mM NaF, 6 mM $MgCl_2$, 3 mM DTT, 10 mM K-PEP, 347 μM [^{14}C]Mtl, 50 mM KP_i, pH 5.4, and no (▼) or 1% lubrol PX (○). Before assaying, the vesicles were incubated for 20 min at 30 °C with (○) or without (▼) 5% lubrol. Unirradiated control values [μM [^{14}C]Mtl-1-P formed/(μg of membrane protein·min)] (▼) 0.4 and (○) 2.3. The vesicles were phosphorylated before irradiation by using E_I, HPr, and PEP.

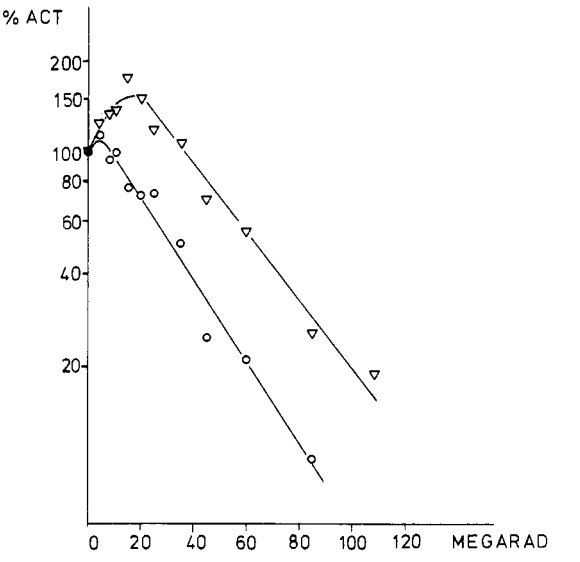

FIGURE 6: Exchange activity measured as a function of radiation dose. Assays (100 μL) were performed at 30 °C in the presence of 40–80 μg of membrane protein, 10 mM NaF, 6 mM $MgCl_2$, 5 mM DTT, 0.12% lubrol PX, 5.5 μM [^{14}C]Mtl, and 800 μM Mtl-1-P: (○) 25 mM KP_i, pH 7.5; (▽) 25 mM NaP_i, pH 6.2. The vesicles were incubated at pH 8.4 with 1% lubrol at 30 °C for 20 min before assaying. Curves are normalized to unit activity at zero radiation dose. Unirradiated control values [nM [^{14}C]Mtl-1-P formed/(μg of membrane protein·min)] were as follows: pH 6.2, 1.5; pH 7.5, 1.2.

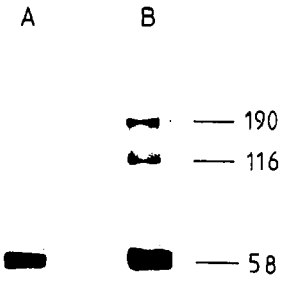

FIGURE 7: Membrane-bound E_{II}^{Mtl} detected by immunoblotting. Membrane proteins were separated on a SDS–7.5 polyacrylamide gel in the absence of mercaptoethanol. For details of immunoblotting see Materials and Methods. Lane A, 10 μg of membrane protein before DTBP treatment; lane B, 10 μg of membrane protein after DTBP treatment.

results in an increase of the amount of "free" dimers, at low pH, in low dose irradiated preparations relative to the unirradiated controls. Consequently, the activity increases relative to these controls. This also accounts for the detergent-induced shift in target volume at pH 8.1, assuming that the detergent shifts the equilibrium between the complexed and uncomplexed E_{II} dimer completely to the uncomplexed state. At that point, one would measure the real target volume responsible for the enzymatic activity.

The above proposal predicts that the interaction between E_{II} and the macromolecule at low pH could also be broken by the use of detergent, but because the association is stronger at low pH, higher concentrations of detergent may be required. Assaying at low pH under the conditions used above still gave curves increasing at low doses followed by a decrease at high doses. Consequently, vesicles were diluted to half the concentration used before but now in the absence or presence of 5% lubrol in 45 mM potassium phosphate, pH 5.4. After incubation, 20-μL samples were used to measure the radiation-dependent activity at pH 5.4 (Figure 5). A sixfold increase of the detergent-treated unirradiated control activity was observed. Furthermore, the initial increase in the low dose irradiated samples was abolished by detergent treatment.

Effect of Irradiation on Exchange Activity. E_{II}^{Mtl} catalyzes the exchange reaction in Scheme II as well as the phosphorylation reaction in Scheme I. Saier (1980) suggested that different functional volumes of E_{II} might be responsible for catalyzing these reactions: a monomer for phosphorylation and a dimer for exchange. Under certain assumptions (no energy transfer between subunits) this could be reflected in the target volume. Therefore, the same vesicles used for PEP-dependent phosphorylation were treated with 1% lubrol, were diluted 2 times, and were used to measure the exchange at pH 6.2 and 7.5. The dose dependence of the activity, plotted in Figure 6, is identical with that measured for PEP-dependent phosphorylation activity. Both the target volume (141 kDa) and the pH-induced increase are similar. This also proves that the observed stimulation effect does not depend on interactions of E_I or HPr with E_{II} but can be attributed to the membrane-bound carrier itself.

Effect of Protein Cross-Linkers on Membrane-Bound E_{II}^{Mtl}. Chemical cross-linking has also been employed to investigate the quaternary structure of the enzyme in situ. Vesicles, isolated in phosphate buffer in the absence of DTT, were diluted in 50 mM NaP_i, pH 8, to a protein concentration of 1 mg/mL. The solution was made 9.2 mM in dimethyl 3,3′-dithiobis(propionimidate) (DTBP) and incubated for 30 min at room temperature. The reaction was terminated by adding ammonium acetate to a concentration of 20 mM. Membrane protein (0.1 mg) was electrophoresed on a 7.5% SDS–polyacrylamide gel under nonreducing conditions. The gel pattern was electrophoretically transferred to nitrocellulose, and E_{II}^{Mtl} was detected by means of immunoblotting (for details see Materials and Methods). Figure 7A shows that, before addition of DTBP, E_{II} only appeared as a monomer on an immunoblot. Since this material was not treated with DTT

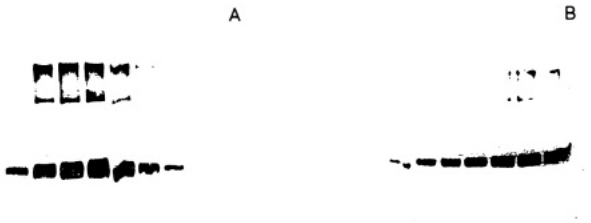

FIGURE 8: Elution of cross-linked E_{II}^{Mtl} containing fractions followed by immunoblotting: (A) hexylagarose column; (B) butylagarose column.

or mercaptoethanol, these data demonstrate that spontaneous oxidation to a dimer did not occur during the course of the experiment. After cross-linking, two additional bands with molecular masses of 116 and ~190 kDa became visible (Figure 7B). The appearance of the two bands depended on both the concentration of the cross-linker used and the reaction time. Although these data are not shown, the higher complex had the tendency to form spontaneously when the vesicles were extracted after dilution in a more acid buffer (50 mM NaP_i, pH 5.0). The molecular mass of the higher component appeared to depend strongly on the percentage acrylamide used in the running gel. The higher the percentage acrylamide, the higher the apparant molecular mass. Values ranged from 148 kDa on 5% polyacrylamide gels to 221 kDa on 8.5% polyacrylamide gels. On the same gels the E_{II} monomer varied from 46 to 49 kDa and the dimer from 102 to 121 kDa.

Analysis of High Molecular Mass Complex with 2D Electrophoresis. Cross-linked EII complexes were purified from the membrane as indicated (Materials and Methods). The E_{II}-containing fractions were monitored by immunoblotting as well as by enzymatic assays to avoid losing E_{II} fractions that had been inactivated by cross-linking. The cross-linked material behaved identically with the un-cross-linked E_{II}^{Mtl} in terms of the extraction procedure and the point of elution from the hexylagarose and butylagarose columns (Figure 8). Moreover, the ratio of cross-linked to non-cross-linked material did not increase during purification, indicating that no significant intermolecular disulfide formation occurred by spontaneous oxidation during the purification (compare Figures 7B and 8A).

The purified cross-linked preparation was subjected to 2D electrophoresis. The first dimension was run on a 1.3 mm thick slab gel under nonreducing conditions. The lane was sliced out and run in the second dimension on a 3 mm thick slab gel with a 1% agarose stacking gel containing 50 mM DTT. Since DTBP contains a disulfide, the cross-link can be broken by reduction. Duplicate gels were run. One was used for silver staining and the other for immunoblotting. If the higher complex was composed of E_{II}^{Mtl} bound to another protein, this protein would appear in the second dimension off the diagonal, staining with silver but not complexing antibodies. The silver-stained gel in Figure 9A shows a large spot (1) at the position of E_{II}^{Mtl} on the diagonal corresponding to non-cross-linked E_{II} and two bands (3 and 5) at the same height as spot 1 but off the diagonal corresponding to E_{II} which had run in the first dimension as a cross-linked, high molecular mass species. The immunoblot in Figure 9B confirms that these protein species are E_{II}^{Mtl}. Residual cross-linked E_{II} (bands 2 and 4) can been seen on the silver-stained gel (A) but is not visible on the immunoblot (B), probably because the concentration is too low. On similar gels, we have sometimes observed minor immunostains at these positions. There is no other band occurring vertical to band 3 or 5 with more than a fraction of the intensity of the E_{II} band.

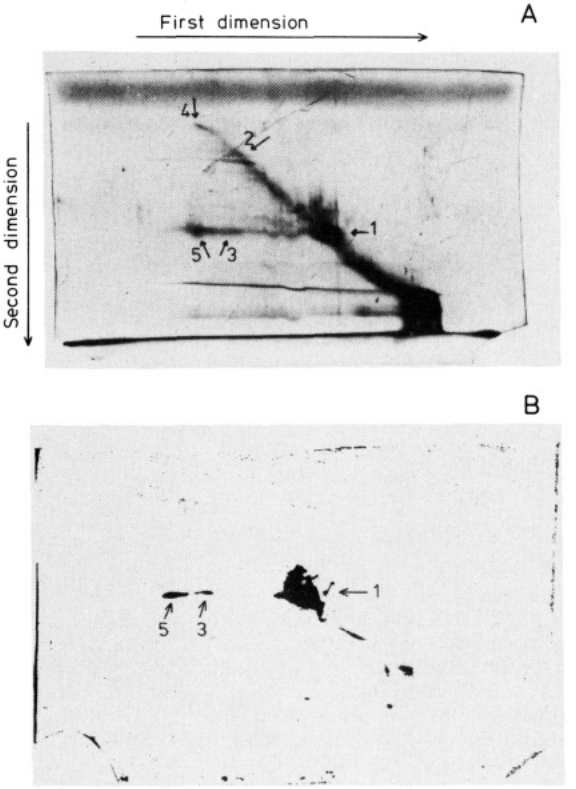

FIGURE 9: Two-dimensional slab gel electrophoretic analysis of cross-linked purified E_{II}^{Mtl}: (A) after silver staining; (B) after immunodetection. E_{II}^{Mtl}-containing spots are indicated by arrows: 1, E_{II} monomer; 2, cross-linked dimer; 3, de-cross-linked dimer; 4, cross-linked oligomer; 5, de-cross-linked oligomer.

DISCUSSION

Kempner and Schlegel (1979) reviewed the target volumes obtained for a number of proteins examined by radiation inactivation. There appeared to be two major classes of results. Either the target volume reflected the molecular mass of the entire protein or it reflected the mass of one or more of the subunits. Thus, radiation inactivation data report on the minimum volume of the functional unit and not necessarily on the volume of the entire complex.

Measurements with purified enzyme suggested that an oligomeric form might be the functionally active species of E_{II}^{Mtl} (Saier, 1980; Roossien et al., 1984; Robillard & Blaauw, 1987). In this report we have examined the quaternary structure of the protein in situ using radiation inactivation and estimated a molecular mass of 140 000 ± 20 000 kDa as the target volume. This value was obtained with vesicles assayed at neutral to slightly basic pH after being treated with a low concentration of lubrol. Detergent treatment was necessary because, in untreated vesicles, a small amount of E_{II} appears to occur in another functional form which influenced the inactivation curves and, thus, the calculated molecular mass. The molecular mass of the E_{II}^{Mtl} monomer, determined from the gene sequence, is 68 000 kDa (Lee & Saier, 1983); consequently, our results indicate that the dimer is the minimum E_{II}^{Mtl} structural unit present in the membrane. If no radiation energy is transferred between the two subunits, the dimer is the functional unit. Energy transfer has been reported for only a few proteins (Houslay et al., 1977; Beauregard et al., 1980; Goldkorn et al., 1984).

Higher Aggregation States. The change in curvature of the inactivation curves upon changing the pH of the assay mixture could have been an artifact of the irradiation process or the membrane preparation itself. Several possibilities were ex-

amined. (i) An acid phosphatase, active at low pH in the control membranes, could lead to low levels of phosphorylation or exchange activity. Destruction of this enzyme by irradiation would produce an apparent stimulation of activity. No phosphatase activity capable of degrading Mtl-1-P could be found in the control membranes. Moreover, the lubrol effect in Figure 4 is inconsistent with this explanation. (ii) A change in the barrier nature of the membrane could explain the increased activity upon irradiation. Inverted vesicles were used in all studies. Active PEP-dependent mannitol transport and phosphorylation requires P–HPr on the outside and Mtl on the inside. Free diffusion of Mtl over the membrane is rate limiting at low Mtl concentration (Lolkema & Robillard, 1985). Irradiation, however, permeabilizes membrane vesicles and could lead to higher activities. The fact that the curves are totally different at low and high pH and that concentrations of lubrol sufficient to permeabilize the membrane do not abolish the stimulation invalidates this explanation. (iii) E_{II} might not be totally destroyed by one hit; a single domain might be destroyed instead. This would leave other domains intact as has been reported by Ottolenghi and Ellory (1983) for the Na^+/K^+-ATPase. If E_{II}^{Mtl} consisted of a phosphorylating and a regulatory domain within one subunit, destruction of the regulatory domain by irradiation should lead to a stimulation of activity upon irradiation of the purified enzyme as well as the membrane-bound enzyme. The abolishment of the low-dose increase at low pH by lubrol already indicates that it is not a question of intramolecular domains. Moreover, when purified enzyme was irradiated, no stimulation under any condition was observed (not shown).

The molecular mass of the purified enzyme cannot be determined by radiation inactivation analysis. The purified enzyme population is an equilibrium between monomers and oligomers, as indicated from exchange experiments (Saier, 1980; Roossien et al., 1984) and PEP-dependent phosphorylation (Robillard & Blaauw, 1987). Irradiation and assay conditions will influence the above equilibrium and thus the target volume.

The nature of the macromolecule that reduces the activity of the E_{II} dimer remains uncertain. We have not been able to visualize a distinct regulator on 2D SDS gels. This could mean that no cross-linking occurs under the stated conditions or that its molecular weight is similar to that of the E_{II} monomer. In this case, the E_{II} monomer itself could also be the third component. Harmon et al. (1980) calculated the molecular weight of the regulatory component of the insulin receptor from the initial increase in their inactivation curves. This required knowing the value of the stimulation factor (S) and the distribution between the complexed and uncomplexed form. Since we do not know the exact values of these parameters, we cannot calculate the size of this protein component. If energy transfer would take place between E_{II} and this component, irradiation at low pH could be a way to obtain the molecular mass of the regulator. The inactivation curve would then give the total molecular mass of the complex as target volume. However, irradiation at pH 5.6 did not result in changing inactivation curves (not shown).

Until now, E_{II} monomers as well as dimers have been reported on SDS–polyacrylamide gels for detergent-extracted membranes (Roossien & Robillard, 1984; Stephan & Jacobsen, 1986a). Detergents, however, strongly influence protein–protein interactions. In Figure 7 we now show that E_{II} can be cross-linked in situ to higher complexes. Analysis of these complexes (Figure 9) showed them to consist of E_{II} only or of E_{II} and an identical molecular mass enzyme. Assuming that only E_{II} is present, we observe in addition to the monomer a dimer and a higher complex. The value of 190 kDa on 7.5% polyacrylamide gel comes close to a trimer. Unfortunately, the molecular mass shows a strong dependence on the percentage acrylamide used. Values ranging from 3 to 4 times that of the monomer were observed. A similar behavior has been seen for oxidized, purified E_{II} and purified E_{II} cross-linked through intersubunit thiols. In these cases, however, the molecular mass extrapolated to twice the monomer value at low polyacrylamide concentrations (Roossien et al., 1986). This was explained by the effect of lubrol on the tertiary structure of the enzyme. In the present study the in situ cross-linked dimer electrophoreses at its correct position.

Independent evidence supporting the existence of a higher aggregated form of E_{II}^{Mtl} can be found in several reports: (i) Stephan and Jacobson (1986b) observed that, after mild trypsinolysis of E_{II} in minicells, the membrane-bound 34-kDa part of the enzyme oligomerized to a 99-kDa SDS–PAGE band; they suggested that it could represent a trimer. Under these same extraction conditions the intact protein behaved as a dimer. (ii) Robillard and Blaauw (1987) report that the specific PEP-dependent mannitol phosphorylation activity of purified E_{II}^{Mtl} increases at low E_{II} concentrations and decreases again at high enzyme concentrations. Further study will be necessary to determine whether these observations are related to the phenomena documented in this report.

Conclusions

E_{II}^{Mtl} is present in the membranes as a dimer. The dimer activity is reduced by a third protein which is able to associate with EII. The activity of the protein is lowered upon association and the association is favored by low pH.

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Tyrosine and Carboxyl Protonation Changes in the Bacteriorhodopsin Photocycle. 1. M_{412} and L_{550} Intermediates†

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ABSTRACT: The role of tyrosines in the bacteriorhodopsin (bR) photocycle has been investigated by using Fourier transform infrared (FTIR) and UV difference spectroscopies. Tyrosine contributions to the $BR_{570} \rightarrow M_{412}$ FTIR difference spectra recorded at several temperatures and pH's were identified by isotopically labeling tyrosine residues in bacteriorhodopsin. The frequencies and deuterium/hydrogen exchange sensitivities of these peaks and of peaks in spectra of model compounds in several environments suggest that at least two different tyrosine groups participate in the bR photocycle during the formation of M_{412}. One group undergoes a tyrosinate → tyrosine conversion during the $BR_{570} \rightarrow K_{630}$ transition. A second tyrosine group deprotonates between L_{550} and M_{412}. Low-temperature UV difference spectra in the 220-350-nm region of both purple membrane suspensions and rehydrated films support these conclusions. The UV spectra also indicate perturbation(s) of one or more tryptophan group(s). Several carboxyl groups appear to undergo a series of protonation changes between BR_{570} and M_{412}, as indicated by infrared absorption changes in the 1770-1720-cm^{-1} region. These results are consistent with the existence of a proton wire in bacteriorhodopsin that involves both tyrosine and carboxyl groups.

Bacteriorhodopsin (bR),[1] the M_r 26 000 integral protein in the purple membrane of *Halobacteria halobium*, functions as a light-driven proton pump (Stoeckenius & Bogomolni, 1982). During this photochemical process, the protein proceeds through a series of intermediates characterized by their visible absorption maxima. Recent evidence indicates that formation of the early photointermediate K involves several molecular events including (i) the isomerization of the all-trans chromophore about the 13-14 double bond (Braiman & Mathies, 1982), (ii) a substantial alteration in the local environment of the protonated Schiff base which is consistent with predicted charge separation from a counterion (Honig et al., 1979; Rothschild et al., 1984), and (iii) the protonation of a tyrosinate ion along with the perturbation of a tryptophan residue (or residues) (Rothschild et al., 1986). Further steps in the

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[1] Abbreviations: $^2H/H$, deuterium/hydrogen; PM, purple membrane; bR, bacteriorhodopsin; [$^2H_4$]Tyr, ring-perdeuteriated L-tyrosine; bR-[$^2H_4$]Tyr, [$^2H_4$]Tyr-incorporated bacteriorhodopsin; FTIR, Fourier transform infrared.

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