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# Specific labeling of the *lac* carrier protein in membrane vesicles of *Escherichia coli* by a photoaffinity reagent

(active transport/nitrophenyl ether/nucleophilic aromatic photosubstitution/triplet state/scavenging)

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ABSTRACT 4-Nitrophenyl-α-D-galactopyranoside (NPG) was used as a photoaffinity reagent to specifically inactivate the β-galactoside transport system in *Escherichia coli* ML 308-225 membrane vesicles. Photolysis of NPG produced time-dependent, irreversible loss of transport activity with corresponding incorporation of [$^3$H]NPG into the membrane. Both processes were blocked by β-D-galactopyranosyl 1-thio-β-D-galactopyranoside, a high-affinity substrate of the *lac* carrier protein, and inactivation of lactose transport was specific because NPG photolysis did not affect proline uptake or the ability of the vesicles to generate an electrochemical proton gradient. Arylation of the *lac* carrier protein was stoichiometric and resulted in the formation of 0.25 nmol of NPG adduct per mg of membrane protein. All attempts to regenerate transport activity by reillumination in the presence of externally added nucleophiles failed, indicating that arylation is functionally irreversible. When vesicles labeled with [$^3$H]NPG under defined experimental conditions were solubilized and analyzed by gel electrophoresis, only one radioactive peak with an apparent molecular weight of 30,000 was observed, confirming that the reaction is highly specific. The results demonstrate that NPG is an active-site-directed photoaffinity label for the *lac* carrier protein.

Bacterial cytoplasmic membrane vesicles have become an increasingly important experimental tool for studying active transport (1, 2). Vesicles prepared from *Escherichia coli*, which have been used most extensively, exhibit essentially the same structure as the membrane in the intact cell (1, 3–6). Moreover, as postulated by Mitchell (7), these vesicles catalyze the accumulation of many different solutes by reactions that utilize an electrochemical gradient of $H^+$ ($\Delta\bar{\mu}_{H^+}$) as the immediate driving force (8–13).

Recent studies focusing on the mechanism of β-galactoside translocation in *E. coli* membrane vesicles indicate that carrier-mediated lactose efflux down a concentration gradient is an ordered reaction in which lactose is released first, followed by deprotonation of the porter (14). Other results (15) suggest that the rate-determining step for turnover involves a reaction that corresponds kinetically to the return of an unloaded, negatively charged porter to the surface of the membrane. More recent analyses (16) demonstrate that in addition to acting thermodynamically as the driving force for active transport, $\Delta\bar{\mu}_{H^+}$ functions in a regulatory capacity by altering the distribution of the *lac* carrier between two distinct kinetic states.

Another approach has been chemical modification of carrier activity, and evidence from one aspect of these studies (17) suggests that a histidyl residue(s) may be involved in the response of the *lac* carrier and other porters to $\Delta\bar{\mu}_{H^+}$. Furthermore, rates of covalent modifications of *lac* carrier activity by diethylpyrocarbonate (17) and by various maleimides (unpublished data) are enhanced in the presence of $\Delta\bar{\mu}_{H^+}$, implying that there may be a structural modification in the *lac* carrier that is the basis for the kinetic alteration observed with this transport system (16). Although little is known about the organization of the *lac* carrier in the membrane, it has been suggested that oligomeric structure (i.e., dimerization) may be essential to its function (16, 18–20).

Another potential means of studying *lac* carrier structure is to label the protein *in situ*; although this has been accomplished (21, 22), the method utilized was not very specific. In this context, affinity-labeling techniques have led to the characterization of several transport systems (23–25), and photoaffinity labeling, in particular, has had relatively widespread use (26). These techniques—specifically, the use of aromatic azides—have been applied to the β-galactoside transport system in this laboratory (27, 28). Although specific photoinactivation of lactose transport with two (2-nitro-4-azidophenyl)-β-D-galactopyranosides has been clearly documented, the long lifetime and reactivity of the nitrenes generated has made radiochemical analysis unsatisfactory (unpublished data).

Recently, Jelenc *et al.* (29) described another class of high-yield photoreagents for affinity labeling. The use of these reagents is based on the nucleophilic aromatic photosubstitution reactions of nitrophenyl ethers, the chemistry of which has been characterized (30, 31). Because 4-nitrophenyl-α-D-galactopyranoside (NPG) is a nitrophenyl ether with a high affinity for the *lac* carrier (32, 33), it was apparent that it might represent a useful reagent for labeling this protein. This communication reports the initial characterization of NPG as an active-site-directed photoaffinity label.

## MATERIALS AND METHODS

**Materials.** NPG, β-galactopyranosyl 1-thio-β-D-galactopyranoside (GalSGal), and lithium D-lactate were purchased from Sigma, [1-$^{14}$C]lactose from Amersham/Searle, and L-[*U*-$^{14}$C]proline from New England Nuclear. [2-$^3$H]NPG was synthesized by Yu-Ying Lui (Isotope Synthesis Group, Hoffmann–La Roche) under the direction of Arnold Liebman and had a specific activity of 347 mCi/mmol (1 Ci = $3.7 \times 10^{10}$ becquerels). Other commercially available compounds were reagent grade.

**Growth of Cells and Preparation of Membrane Vesicles.**

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Abbreviations: NPG, 4-nitrophenyl-α-D-galactopyranoside; GalSGal, β-galactopyranosyl 1-thio-β-D-galactopyranoside; PMS, phenazine methosulfate; $\Delta\Psi$, membrane potential; $\Delta\bar{\mu}_{H^+}$, electrochemical gradient of $H^+$; ΔpH, chemical gradient of $H^+$.

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*E. coli* ML 308-225 ($i^-z^-y^+a^+$) and ML 30 ($i^+z^+y^+a^+$) were grown on minimal medium with 1.0% disodium succinate (hexahydrate), and membrane vesicles were prepared and stored as described (3, 34).

**Transport and [$^3$H]NPG-Labeling Assays.** Transport was measured with reduced phenazine methosulfate (PMS) as electron donor (35). Incorporation of [$^3$H]NPG was monitored by diluting 0.2 ml of sample containing 1 mg of protein/ml into 5.0 ml of cold 10% trichloroacetic acid and mixing vigorously. After incubation on ice for 15 min, the precipitated material was collected by filtration on 47-mm Amicon filters (0.45 $\mu$m pore size) and washed twice with 5.0 ml of cold trichloroacetic acid. The filters were then assayed directly by liquid scintillation techniques with a typical efficiency of 22%.

**Photolysis Conditions.** Photoinactivation and labeling studies were carried out in a jacketed tube (1.0 cm internal diameter) maintained at 25°C by circulating water. The sample (up to 5.0 ml) was stirred with a magnetic stirrer and, where indicated, flushed with a stream of water-saturated argon. Samples were illuminated with a mercury arc lamp (100 W, type AH-4) with a Corning 0-54 filter to cut off light below 300 nm.

**Other Techniques.** NaDodSO$_4$/7.5–20% polyacrylamide gradient gel electrophoresis by the Laemmli buffer system (36) was used routinely. Protein was measured by the method of Lowry *et al.* (37). Spectral recordings were made with an Aminco DW-2 spectrophotometer. Measurements of membrane potential ($\Delta\Psi$) and pH gradient ($\Delta$pH) were performed by flow dialysis using appropriate probes (15).

## RESULTS

**Photolysis of NPG.** Before testing NPG as a substrate for photoinactivation of the $\beta$-galactoside transport system, preliminary experiments were performed to examine its photolysis characteristics (Fig. 1). The nitrophenyl chromophore of NPG exhibits an absorbance maximum at 305 nm; after exposure to light above 300 nm under mildly alkaline conditions, there is time-dependent appearance of a second absorbance maximum at 400 nm. The second maximum is characteristic of *p*-nitrophenylate ion, the product expected from the reaction of NPG with hydroxide ion, and after 1 hr under the conditions described, approximately 50% of the NPG is hydrolyzed.

When the reaction was carried out under the same conditions with a solution containing bovine serum albumin (1 mg/ml) and 80 $\mu$M [$^3$H]NPG, incorporation into trichloroacetic acid-precipitable material was linear with time, resulting in approximately 3 mol of *p*-nitrophenyl adduct per mol of albumin after 2 hr of photolysis (data not shown). Thus, NPG can serve as a photolabel, albeit nonspecifically, for albumin.

**NPG Photolysis Inactivates Lactose Transport Specifically.** In order to determine whether NPG can be used as an active-site-directed inactivator of the *lac* carrier protein, the transport activity of *E. coli* ML 308-225 membrane vesicles was monitored after illumination for various times in the presence of NPG. A vesicle suspension was placed in the reaction vessel, NPG was added to 20 $\mu$M [a concentration approximately 3 times the $K_d$ (32, 33)], and, before initiation of photolysis, D-lactate was added and the suspension was stirred under argon to achieve anaerobiosis (the requirement for these conditions is described below). After illumination for given periods of time, samples were removed, washed twice by centrifugation, and assayed for lactose transport (Fig. 2*A*). Clearly, both the initial rate of lactose transport and the steady-state level of accumulation were diminished progressively after 10, 30, and 60 min of illumination; after 60 min, lactose transport was completely abolished. Importantly, however, when 10 mM GalSGal, another high-affinity substrate for the *lac* carrier, was included in the reaction mixture during illumination, no inhibition was observed. Furthermore, no inhibition was observed when illumination was carried out in the absence of NPG, thus ruling out nonspecific effects of light. Independent measurements (not shown) demonstrated that the illumination under the conditions described did not impair either substrate oxidation or the generation of $\Delta\Psi$ and $\Delta$pH via reduced PMS oxidation. Loss of $\beta$-galactoside transport activity was irreversible because repeated washing of the vesicles after photolysis (up to 10 times) failed to restore activity (data not shown). Finally, the rate of NPG photoinactivation was concentration dependent, and longer photolysis times were required for inactivation when NPG was present at concentrations below the $K_d$ (6 $\mu$M).

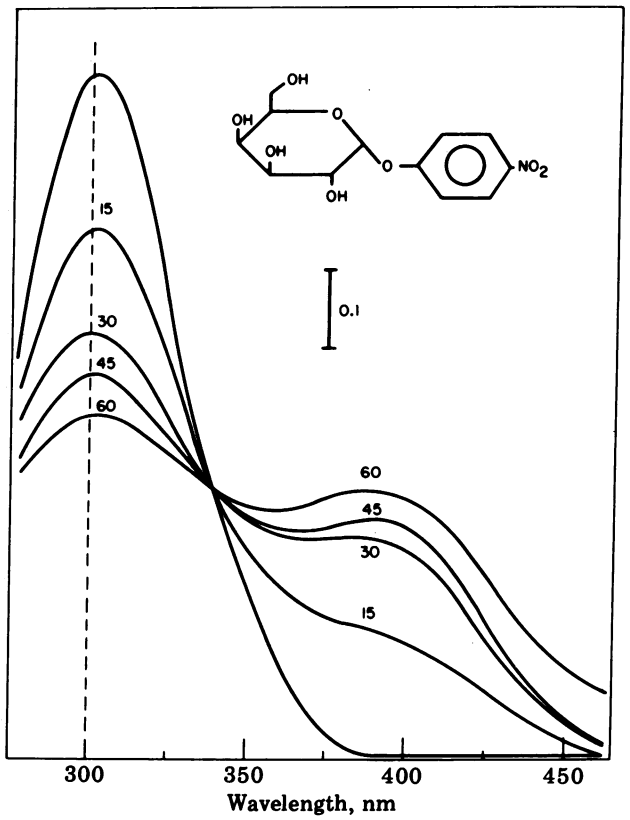


FIG. 1. Photoinduced hydrolysis of NPG. An 80 $\mu$M solution of NPG in 0.1 M sodium phosphate (pH 8.0) was prepared by appropriate dilution from an NPG stock solution in ethanol (20 mM). After the absorbance spectrum was recorded, the solution was photolyzed; at given times, a 1.0-ml aliquot was removed from the photolysis chamber and its spectrum was recorded. Spectra taken at 0 time and after 1 hr in the dark were identical. Vertical dashed line, cutoff filter; vertical scale, 0.1 absorbance unit.

The specificity of NPG photoinactivation was further demonstrated with vesicles that were illuminated for 30 min in the presence of NPG [i.e., under conditions such that lactose transport was inactivated by 65% (Fig. 2*A*)] and then assayed for proline transport (Fig. 2*B*). There was no appreciable difference in proline transport activity between vesicles illuminated in the presence or absence of NPG and those that were not illuminated.

**Specificity and Stoichiometry of Labeling.** The specificity of photolabeling was studied with [$^3$H]NPG by labeling vesicles under various conditions (Fig. 3). With 20 $\mu$M [$^3$H]NPG, incorporation of radioactivity increased with time and did not level off even after 90 min, when about 1.0 nmol of adduct was formed per mg of membrane protein. Although significantly less incorporation was observed in the presence of GalSGal,

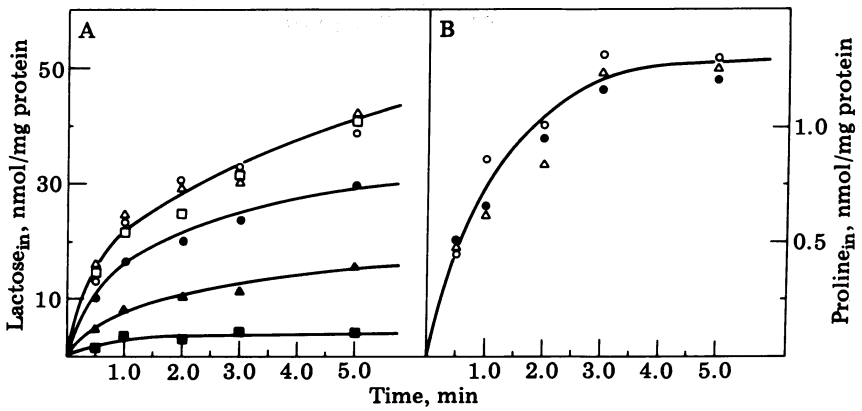


FIG. 2. Effect of NPG photolysis on transport of lactose (*A*) and proline (*B*). (*A*) *E. coli* ML 308-225 vesicles were resuspended to 1.0 mg of protein/ml in 0.1 M potassium phosphate (pH 6.6), and 5.0 ml was placed in a photolysis cell. The suspension was gassed with argon for 3 min in the dark and D-lactate was added to a final concentration of 20 mM. After another 3 min, NPG was added to 20 $\mu$M final concentration and illumination was begun immediately. At various times, the vesicle suspension was removed from the chamber, washed twice by centrifugation with 0.1 M potassium phosphate (pH 6.6), and resuspended to a final concentration of 4.0 mg of protein/ml. Aliquots (15 $\mu$l) were diluted to a final volume of 50 $\mu$l containing (in final concentrations) 50 mM potassium phosphate (pH 6.6) and 10 mM magnesium sulfate and assayed for lactose uptake in the presence of potassium ascorbate (20 mM) and PMS (0.1 mM) in a water-saturated oxygen atmosphere. [1-$^{14}$C]Lactose (57 mCi/mmol) was used at 0.4 mM. Vesicles were illuminated in the presence of NPG for 10 min (●), 30 min (▲), or 60 min (■). Vesicles treated in the same fashion without illumination (○), illuminated for 60 min in the absence of NPG (□), or illuminated for 60 min with NPG and 20 mM GalSGal (△) also were assayed for transport activity. (*B*) Vesicles were illuminated in the presence of NPG as described in *A* and assayed for ascorbate/PMS-dependent proline uptake with [*U*-$^{14}$C]proline (248 mCi/mmol) at a final concentration of 8 $\mu$M. Proline transport in unilluminated control vesicles (△) and vesicles illuminated in the absence of NPG for 30 min (○) or illuminated in the presence of 20 $\mu$M NPG for 30 min (●) were assayed.

appreciable time-dependent labeling was observed, indicating that a reasonably large fraction of the reaction was nonspecific under these conditions. This indication was confirmed by experiments carried out with 85 $\mu$M NPG, a concentration far in excess of the $K_d$ of the *lac* carrier for this substrate. At this concentration, the rate of labeling increased approximately 5-fold.

Importantly, most nonspecific labeling was prevented when the photolysis reaction was carried out anaerobically in the presence of an oxidizable substrate such as D-lactate.[§] This was illustrated initially in the labeling experiments with 85 $\mu$M [$^3$H]NPG. After addition of D-lactate at 10 min (arrow), rapid incorporation of label continued for another 2.5 min, until the oxygen concentration in the photolysis chamber became limiting and the rate of incorporation decreased sharply (Fig. 3). Although not shown, maximal reduction of nonspecific labeling was achieved under anaerobic reducing conditions (i.e., gassing with argon in the presence of D-lactate). Nonspecific labeling still was observed when photolysis was carried out under oxygen in the presence of D-lactate or under argon in the absence of an oxidizable substrate. Under anaerobic reducing conditions with 20 $\mu$M [$^3$H]NPG, total labeling was decreased to less than 50% (Fig. 3; compare solid boxes with solid circles), and approximately 60% was blocked by GalSGal (compare open and solid boxes).

Plotting the difference in incorporation in the absence and presence of GalSGal as a function of illumination time (Fig. 3 *Inset*) showed that incorporation saturated with time, and a maximum of about 0.25 nmol of adduct was formed per mg of membrane protein,[¶] in close agreement with the amount of *lac* carrier in the membrane as judged by other criteria (33, 38). It is also noteworthy that complete arylation occurs in about 1 hr, which correlates well with the inactivation data presented in Fig. 2.

In the experiments presented in Fig. 4, vesicles were photolabeled with 20 $\mu$M [$^3$H]NPG under anaerobic reducing conditions and then subjected to NaDodSO$_4$/polyacrylamide gel electrophoresis. Although many proteins appeared in the gel, only one major radioactive band was observed; this band migrated with an apparent molecular weight of 30,000, which is in excellent agreement with other studies on the *lac* carrier protein (20–22). In contrast, when photolabeling was performed with vesicles prepared from uninduced *E. coli* ML 30 (i.e., having no *lac* carrier) or with ML 308-225 vesicles in the presence of 20 mM GalSGal, the radioactivity profile was essentially flat. Thus, the radioactive band in Fig. 4*B* represents the *lac* carrier protein. When photolabeling was carried out with ML 308-225 vesicles under aerobic conditions, approximately 10 radioactive bands were observed, of which only 1 migrated at 30,000 daltons (data not shown). Therefore, when the photolabeling reaction is performed under anaerobic reducing conditions, most of the nonspecifically reactive [$^3$H]NPG is "scavenged."

Recovery of radioactivity specifically associated with the *lac* carrier in a number of experiments such as that shown in Fig. 4*B* was only 20–25% at most. For this reason, preliminary stability studies were undertaken in order to study the possibility of partial noncovalent interactions between NPG and the *lac* carrier or breakdown of the adduct. Vesicles were photolabeled as described in Fig. 4*B*, washed extensively, and reilluminated for up to 1 hr in the presence of various nucleophiles (methylamine, hydroxylamine, or dithiothreitol at a final concentration of 10 mM) or excess unlabeled NPG (1.6 mM). There was no significant loss of radioactivity under any of these conditions after precipitation with cold 10% trichloroacetic acid (data not shown).

## DISCUSSION

Based on the following criteria (39), NPG has been shown to be an active-site-directed photoaffinity label for the *lac* carrier protein in *E. coli* membrane vesicles: (*i*) NPG is a potent competitive inhibitor of lactose transport and binds specifically to the *lac* carrier protein (32, 33); (*ii*) during illumination, there

[§] Although D-lactate was routinely used, the effect was not specific for this electron donor. Several other substrates (L-lactate, succinate, and reduced PMS) also prevented nonspecific labeling under anaerobic conditions.

[¶] Range of 0.25–0.35 nmol of adduct formed per mg of membrane protein, depending on the preparation of vesicles used.

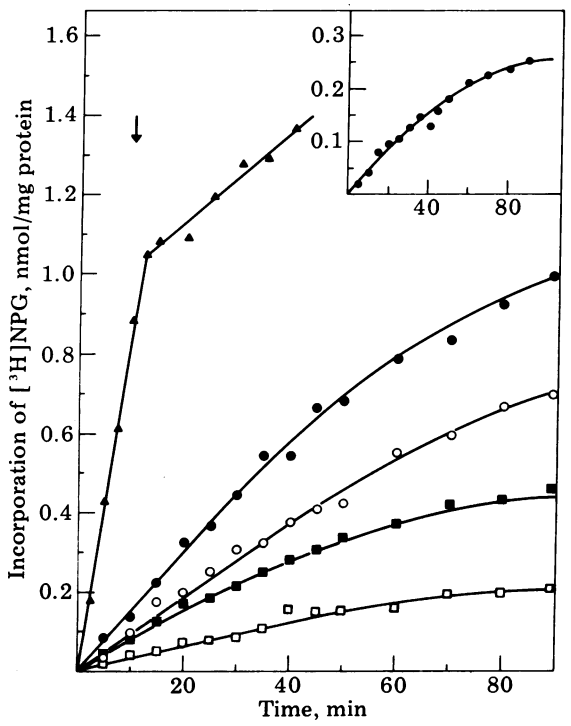


FIG. 3. Incorporation of [³H]NPG into *E. coli* ML 308-225 membrane vesicles. Vesicles were resuspended to 1.0 mg of protein/ml and 5.0 ml was placed in the photolysis cell. [³H]NPG (347 mCi/mmol) was added to 20 μM and illumination was begun. At various times, 200-μl aliquots were removed and mixed vigorously with 5.0 ml of cold 10% trichloroacetic acid. Precipitated protein was collected by filtration and assayed for radioactivity by liquid scintillation techniques (●). The same experiment was repeated in the presence of 20 mM GalSGal (○). Vesicles were also photolyzed with 85 μM [³H]NPG and incorporation was monitored with time (▲); at 10 min (arrow), D-lactate (20 mM) was added and the reaction was continued. In another experiment, vesicles were preequilibrated with an argon atmosphere and 20 mM D-lactate (as described in Fig. 2) before photolysis was initiated. The time course of [³H]NPG incorporation was then determined in the absence (■) or presence (□) of 20 mM GalSGal. (*Inset*) Plot of the difference in labeling observed in the absence and presence of GalSGal (in nmol/mg of protein) as a function of time (min).

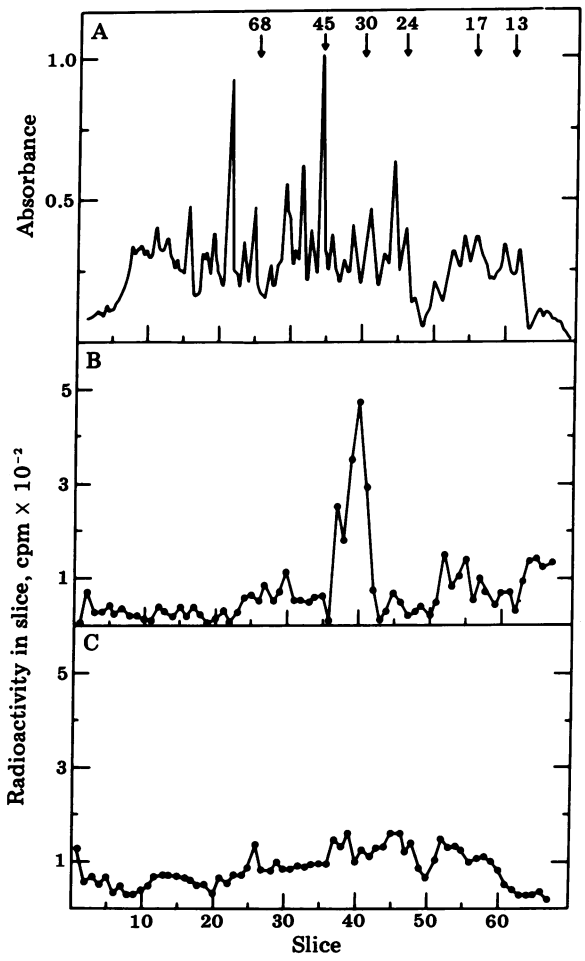


FIG. 4. Analysis of [³H]NPG-labeled membrane proteins by $NaDodSO_4$/polyacrylamide gel electrophoresis. *E. coli* ML 308-225 vesicles were illuminated for 10 min with [³H]NPG (20 μM) in the presence of argon and D-lactate as in Fig. 3. The vesicles were then collected by centrifugation, washed twice with 0.1 M sodium phosphate (pH 6.6), and concentrated to ≈15–20 mg of protein/ml. An aliquot (50 μl) of the sample was added to an equal volume of 125 mM Tris·HCl, pH 6.8/6% $NaDodSO_4$/20% glycerol/1 mM EDTA (Na salt), and the resulting mixture was incubated at 37°C for 30 min. Samples (25 μl) were then subjected to electrophoresis on a $NaDodSO_4$/7.5–20% polyacrylamide gradient gel along with appropriate molecular weight standards (shown by arrows at top, $\times 10^{-3}$). (*A*) Part of the gel was stained with Coomassie brilliant blue G, destained, and photographed; a densitometric scan of the negative is shown. (*B*) Part of the gel was cut into 1-mm slices. Each slice was digested in a scintillation vial containing 0.4 ml of 60% perchloric acid and 0.8 ml of 30% hydrogen peroxide for 12 hr at 65°C and analyzed by liquid scintillation techniques using Dimiscint (National Diagnostics) as the scintillation cocktail. Samples were dark adapted before counting to minimize chemiluminescence. (*C*) ML 308-225 vesicles were labeled with [³H]NPG exactly as described, but 20 mM GalSGal was also present in the reaction mixture. ³H incorporation was analyzed as in *B*. An identical labeling pattern was observed with vesicles prepared from *E. coli* ML 30 that were not induced for the *lac* operon.

is time- and concentration-dependent inactivation of lactose transport; (*iii*) loss of transport activity is irreversible; (*iv*) inactivation is specific because proline uptake, substrate oxidation, and the generation of $\Delta\Psi$ and $\Delta pH$ remain intact; (*v*) inactivation of lactose transport correlates stoichiometrically with the incorporation of [³H]NPG into the *lac* carrier under specified experimental conditions; and (*vi*) inactivation of lactose transport and incorporation of radioactivity are blocked by GalSGal, another high-affinity substrate of the *lac* carrier.

Light-induced reactions of nitrophenyl ethers typically proceed by nucleophilic aromatic photosubstitution, resulting in covalent incorporation of the nitrophenyl moiety with elimination of the other portion of the molecule (29–31). The hydrolysis data presented in Fig. 1 provide some evidence that NPG reacts in this fashion. In order to monitor protein arylation, therefore, phenyl-labeled NPG is required, and preliminary photolabeling data with both bovine serum albumin and vesicles suggest that the reactions proceed as expected with incorporation of the nitrophenyl moiety.

NPG was utilized as a photolabel because it binds tightly to the *lac* carrier protein (32, 33) and also because of the advantageous photochemical characteristics of other nitrophenyl ethers (29–31). Presumably, such compounds react via an extremely short-lived triplet state (half-life, 0.01–1 nsec) that decays to ground state upon nonproductive deactivation; depending on the type and concentration of the attacking nucleophile, the chemical yield of the reaction can be high. Although these properties should give rise to highly specific photoreactions, it is apparent that NPG labels a number of *E. coli* membrane proteins in addition to the *lac* carrier. Strikingly, however, most of the nonspecific reactivity is abolished under anaerobic reducing conditions, and the following explanation is suggested. In addition to binding to the *lac* carrier protein,

NPG partitions nonspecifically into the membrane because of the hydrophobic properties of the nitrophenyl group. Thus, during photoactivation under aerobic conditions, the compound reacts both specifically and nonspecifically. If the activated triplet state can accept electrons from the membrane-bound respiratory chain and thus be transformed into an inactive species, however, the nonspecific reactions would be quenched under anaerobic reducing conditions. On the other hand, those NPG molecules buried in the active site of the *lac* carrier might be "insulated" from the respiratory chain and react with a nucleophile(s) in the immediate vicinity, resulting in specific labeling of the carrier. Although chemical evidence regarding such a scheme must await characterization of the low molecular weight products formed during NPG photolysis under anaerobic reducing conditions, the observation that photolysis must be carried out in the presence of an oxidizable substrate *and* under anaerobic conditions to avoid nonspecific labeling is consistent with this idea.

Barring technical problems associated with the ability of the *lac* carrier to aggregate during $NaDodSO_4$/polyacrylamide gel electrophoresis, it should now be possible to examine the oligomeric structure of this protein *in situ* by means of cross-linking studies. In any case, the results presented here provide an encouraging demonstration that this type of photochemistry can be useful in the design of other active-site-directed photoaffinity labels (29).

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