Labelling of the Cyclitol Permease in *Klebsiella aerogenes*

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The protein component(s) of the cyclitol-transport system in *Klebsiella aerogenes* has been labelled by using three different procedures. One method is based on differential alkylation with *N*-ethylmaleimide, and another on incorporation of amino acids during the induction process. A protein of mol.wt. 34 000 was labelled by both procedures; by alkylation with *N*-ethylmaleimide two other proteins of mol.wts. 55 000 and 67 000 were also labelled. The third method uses diazotized [³⁵S]sulphanilic acid after protection by substrate and the comparison of labelling of induced cells with non-induced cells; the label was also concentrated in a mol.wt.-33 000 peak. The labelled protein is, from the evidence, the cyclitol carrier.

The protein components involved in bacterial-transport processes have been extensively studied. In the case of the phosphoenolpyruvate phosphotransferase system the two enzymes and the HPr protein have been purified and isolated (for review see Kundig, 1974). In the osmotic-shock-sensitive transport systems several binding proteins have been isolated and purified to homogeneity and their binding properties are well known (for review see Boos, 1974). Quite different is the picture in the osmotic-shock-insensitive transport systems; since the early *N*-ethylmaleimide labelling experiments carried out by Fox & Kennedy (1965), Carter *et al.* (1968) and Jones & Kennedy (1969) and also since Stein's dual-labelling experiment of the lactose permease (Kolber & Stein, 1966), very few bacterial membrane proteins associated with transport processes have been identified (Fournier & Pardee, 1974; Hirata *et al.*, 1976; Lo & Sandwall, 1975).

The cyclitol-transport system in *Klebsiella aerogenes* has been characterized in our laboratory. It is not sensitive to osmotic shock and neither is it a phosphoenolpyruvate phosphotransferase system (Deshusses & Reber, 1972, 1977; Reber *et al.*, 1977). Specific labelling of the permease has been achieved on the basis of the observation that (i) the system is inducible (Deshusses & Reber, 1972) and (ii) *myo*-inositol prevents the alkylation by *N*-ethylmaleimide when present in a 50-fold excess (Deshusses & Reber, 1974).

Materials and Methods

Bacterial strain, growth conditions and transport assays have been described previously (Deshusses & Reber, 1972).

Abbreviation used: SDS, sodium dodecyl sulphate.

Labelling with amino acids during the induction

Glucose-grown cells were used to inoculate three flasks each containing 5 ml of culture media. Two of the flasks contained 1% (w/v) D-glucose as carbon source and the third 1% (w/v) *myo*-inositol. The absorbance was monitored with a Klett–Summerson (Philadelphia, PA, U.S.A.) photometer with filter no. 54. When the turbidity reached 100 Klett units, 25 μ Ci of L-[U-¹⁴C]phenylalanine (sp. radioactivity 485 mCi/mmol) and 25 μ Ci of L-[U-¹⁴C]leucine (sp. radioactivity 330 mCi/mmol) were added to the *myo*-inositol-containing medium, and 60 μ Ci of L-phenyl[2,3-³H]alanine (sp. radioactivity 16.6 Ci/mmol) and 60 μ Ci of L-[4,5-³H]leucine (sp. radioactivity 60 Ci/mmol) were added to one of the flasks containing D-glucose-supplemented medium, whereas the second D-glucose-containing medium received the same amount of ³H- and ¹⁴C-labelled amino acids. In some experiments, mixtures of 15 radioactively labelled amino acids that were ³H- and ¹⁴C-labelled were used.

The three flasks were incubated in a rotary shaker at 30°C until the absorbance reached 350 Klett units.

N-Ethylmaleimide labelling

The method used is similar to that described by Fox & Kennedy (1965), Carter *et al.* (1968) and Jones & Kennedy (1969). The reaction was carried out in 0.1 M-sodium phosphate/potassium phosphate buffer, pH 6.8.

After harvesting, induced cells were washed three times in the buffer and resuspended in three batches.

(a) *Protected cells.* *myo*-Inositol was added to a final concentration of 50 mM and 30 s later non-labelled 1 mM-*N*-ethylmaleimide was added. The reaction was stopped by centrifugation after 7 min of treat-

ment and the cells were washed five times. 1 mM-*N*-ethyl[2,3- 14 C]maleimide (sp. radioactivity 8.4 mCi/mmol) was added and allowed to react for 7 min. The cells were then carefully washed five times in the buffer.

(b) *Unprotected cells.* This batch was treated only with 1 mM-*N*-ethyl[2- 3 H]maleimide (sp. radioactivity 20 mCi/mmol) for 7 min and washed.

(c) *Control cells.* This batch was treated with 1 mM-*N*-ethyl[2- 3 H]maleimide (sp. radioactivity 20 mCi/mmol) plus 1 mM-*N*-ethyl[2,3- 14 C]maleimide (sp. radioactivity 8.4 mCi/mmol) for 7 min and washed. The protected and unprotected cells were pooled.

Labelling with *p*-diazobenzenesulphonic acid

p-[35 S]sulphanilic acid (The Radiochemical Centre, Amersham, Bucks., U.K.) was diazotized by the method of Berg & Hirsch (1975). Cells were treated with 1 mM-*p*-diazobenzene[35 S]sulphonic acid (sp. radioactivity 200 μ Ci/ μ mol) for 30 min at 30°C in 0.1 M-sodium phosphate/potassium phosphate buffer, pH 7.5. The cells were then centrifuged at 5000 *g* for 10 min and washed three times with the same buffer by centrifugation.

Membrane isolation

The cells were resuspended in 5 ml of 0.1 M-sodium phosphate/potassium phosphate buffer, pH 7.0, and disrupted with a French press (Aminco, Silver Spring, MA, U.S.A.) at a pressure of 1500 kg/cm². The homogenates were centrifuged at 5000 *g* to remove unbroken cells. The supernatant was then centrifuged at 100 000 *g* for 1 h. The supernatant was carefully discarded and the pellet was not washed. Protein determinations were performed by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Polyacrylamide-gel disc electrophoresis

Polyacrylamide gels (10%) were prepared by the method of Laemmli (1970). Electrophoresis was carried out in SDS with a constant current of 2 mA/tube. Bovine serum albumin, ovalbumin and lysozyme were pretreated with SDS and β -mercaptoethanol and used as standards. Molecular-weight-calibration gels were stained for 1 h at 70°C in 0.15% Coomassie Blue in 10% acetic acid and destained electrophoretically in 7% acetic acid plus 10% methanol.

Radioactivity determination in the gels

The radioactive gels were sectioned by using a Gilson (Villiers-le-Bel, France) Gel Fractioner into 1 or 2 mm slices. The fractions were counted for radioactivity in a Beckman LS 255 scintillation counter (Beckman, Palo Alto, CA, U.S.A.) equipped with an AQC system. The scintillation mixture was

toluene/Triton X-100/water (100:50:4, by vol.) supplemented with 10 g of 5-(biphenyl-4-yl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole/litre (Ciba-Geigy, Basel, Switzerland).

Calculations

After corrections for quenching and radioactive-isotope overlapping the total counts for each isotope were calculated. Distribution of the percentage of radioactivity through the gel for each isotope was plotted. Control cells (which had received both isotopes) were used to check the uniformity of isotope distribution.

Chemicals

N-ethyl[2- 3 H]maleimide, *N*-ethyl[2,3- 14 C]maleimide and a mixture of 3 H- and 14 C-labelled amino acids was purchased from New England Nuclear (Dreieichenhain, Germany). The Radiochemical Centre supplied *scyllo*-[2- 3 H]inositol, L-[U- 14 C]-phenylalanine, L-phenyl[2,3- 3 H]alanine, L-[U- 14 C]-leucine and L-[4,5- 3 H]leucine. The other chemicals were obtained from Merck (Darmstadt, Germany) and Fluka (Buchs, Switzerland).

Results

Labelling with amino acids during the induction

The radioactivity present in the membrane preparations ranged between 4.5×10^6 and 1.3×10^7 c.p.m./mg of protein. The distribution of the percentage of radioactivity in the gel slices of the mixed cultures is shown in Fig. 1, and that for the control cells is shown in Fig. 2. In the six experiments only peaks corresponding to mol. wts. 34 000, 45 000 and 74 000 exceeded the 'noise' level. The major significant en-

Fig. 1. Percentage distribution of radioactivity in SDS/polyacrylamide-gel electrophoresis of membrane proteins after radioactively labelled amino acid incorporation

...., 3 H-labelled non-induced cells; —, 14 C-labelled *myo*-inositol-induced cells. The numbers above the curves represent molecular weights of proteins.

Fig. 2. Percentage distribution of radioactivity in SDS/polyacrylamide-gel electrophoresis of membrane proteins from non-induced cells that received both isotopes simultaneously (control cells) —, ^{14}C radioactivity; ····, ^3H radioactivity.

richment of the $^{14}\text{C}/^3\text{H}$ ratio occurs at a relative migration of 0.63. The molecular weight of this protein is 34 000 (isotopic ratio 1.47 ± 0.16). The incorporated radioactivity of the other ^{14}C -enriched minor peaks is very low. Their mol.wts. are 45 000 and 74 000 (isotopic ratios 1.21 ± 0.16 and 1.30 ± 0.24 respectively), which correspond to the main ^{14}C -enriched peaks in the supernatant fraction of the last centrifugation. It is therefore most likely that contamination by the supernatant accounts for these peaks. The pattern of protein labelling varied only slightly from one experiment to another (for six experiments). In every case the mol.wt.-34 000 peak was present with similar intensity, whereas the mol.wt.-74 000 and -45 000 peaks were absent in one experiment. The same labelling pattern was observed with the mixture of 15 radioactive amino acids.

Labelling with *N*-ethylmaleimide

Experiments were performed to determine the duration of exposure of cells to the non-radioactive 1 mM-*N*-ethylmaleimide and the protective effect of the substrate. The activity of the transport system was then tested by measuring the uptake of 0.11 mM-*scyllo*-inositol during 0.5 min. As shown in Fig. 3, 5 min exposure to *N*-ethylmaleimide is sufficient to decrease the uptake by 80% of that of the control cells. Longer exposure is not necessary. The cells protected by 50 mM-*myo*-inositol during *N*-ethylmaleimide exposure retain 70% of the initial uptake value, whereas the amount of cyclitol transported during 2 min begins to decrease after 10 min exposure.

The incorporation of radioactivity into membrane proteins is about 50 times lower than that observed with the induction method. It varies from 2×10^5 to 5.5×10^5 c.p.m./mg of protein. The accuracy of the method is thus limited by the amount of

Fig. 3. Protective effect of *myo*-inositol against *N*-ethylmaleimide alkylation

myo-Inositol induced cells were incubated for the indicated time with 1 mM-ethylmaleimide (broken line) or with 1 mM-*N*-ethylmaleimide plus 50 mM-*myo*-inositol (solid line). After *N*-ethylmaleimide and *myo*-inositol removal by repeated centrifugation, transport activity was assayed with 0.1 mM-*scyllo*-inositol for 0.5 min.

Fig. 4. Percentage distribution of ^3H and ^{14}C radioactivity in SDS/polyacrylamide-gel electrophoresis of membrane proteins from non-protected cells which received a mixture of both isotopically labelled *N*-ethylmaleimides ····, *N*-Ethyl[2- ^3H]maleimide; —, *N*-ethyl[2,3- ^{14}C]maleimide.

protein-bound radioactivity that can be submitted to the gel disc electrophoresis, so that at least five electrophoretic runs were performed for each labelling experiment.

Fig. 4 shows the percentage distribution of *N*-ethyl[2- ^3H]maleimide and *N*-ethyl[2,3- ^{14}C]maleimide-membrane protein through the gel of an un-protected culture (control). Fig. 5 shows the percentage distribution through another gel of a mixture of membrane proteins from ^{14}C -labelled (protected) culture and a ^3H -labelled (non-protected) culture. Three peaks showed significant increase in the $^{14}\text{C}/^3\text{H}$ ratio. The major enriched peak corresponds to a mol.wt. of 34 000 (isotopic ratio 1.25 ± 0.12). The two other enriched peaks also observed correspond to mol.wts. of 55 000 and 67 000 (isotopic ratios 1.17 ± 0.07 and 1.1 ± 0.04 respec-

Fig. 5. Percentage distribution of ^3H and ^{14}C in SDS/polyacrylamide-gel electrophoresis of membrane proteins

...., Unprotected cells labelled with N -ethyl[2- ^3H]maleimide; —, protected cells labelled with N -[^{14}C]ethylmaleimide. The numbers in the Figure represent molecular weights of proteins.

tively). These two peaks were always present in all gels, but their relative intensity varied from one experiment to another. In contrast with the amino acid labelling, contamination by the soluble fraction does not account for these peaks, since the radioactivity in the supernatant was found to be negligible.

Other labelling experiments have been performed to test the influence of the length of the exposure to the non-radioactive N -ethylmaleimide on the labelling pattern of the proteins. The profile of the radioactivity distribution does not vary significantly from the experiment shown. As pointed out by Fournier & Pardee (1974) the labelling of membrane proteins not directly involved in translocation can be explained by the relatively short exposure to the non-radioactive N -ethylmaleimide, and, as a consequence, by the non completion of the first alkylation; therefore other thiol-containing proteins are afterwards labelled. However, longer exposure to the non-radioactive N -ethylmaleimide had to be avoided to preserve the protective effect of the substrate.

Labelling with p -diazobenzene[^{35}S]sulphonic acid

Induced and non-induced cells were treated separately with 1 mM- p -diazobenzene[^{35}S]sulphonic acid as described in the Materials and Methods section. The radioactivity recovered was 1 nCi/ μg of membrane proteins, which represented 2% of the reagent added. Fig. 6 shows the pattern of distribution of the radioactivity through the gels from membrane of induced and non-induced cells, as a function of the relative migration. One peak shows a significant increase with a relative migration corresponding to mol.wt. 33 000.

The effect of the natural substrate of the transport system, myo -inositol, on labelling has been tested by comparison between cells treated as above

Fig. 6. Percentage distribution of radioactivity in SDS/polyacrylamide-gel electrophoresis of membrane proteins labelled with p -diazobenzene[^{35}S]sulphonic acid —, myo -Inositol-grown cells (induced cells);, glucose-grown cells (non-induced cells). The separation was carried out on separate gels so the abscissa is expressed as the relative migration towards the dye. The number above the peak corresponds to molecular weight.

Fig. 7. Percentage distribution of radioactivity in SDS/polyacrylamide-gel electrophoresis of membrane proteins labelled with p -diazobenzene[^{35}S]sulphonic acid myo -Inositol-grown cells (induced) were labelled in the presence (protected cells; —) or in the absence (unprotected cells;) of 0.1 M- myo -inositol. The separation was carried out on separate gels so the abscissa is expressed as the relative migration towards the dye. The number above the peak corresponds to molecular weight.

in the absence or in the presence of 100 mM- myo -inositol. Fig. 7 shows that in the presence of myo -inositol (broken line) the labelling of the mol.wt.-33 000 protein is significantly decreased, compared with the cells (solid line) treated in the absence of the substrate.

Discussion

Three different methods have been used to label the protein components of the myo -inositol-transport system in *K. aerogenes*. These methods all result in the labelling of a mol.wt.-33 000–35 000

protein, which we assume to be a part of the cyclitol-carrier protein. In our system the specific and significant labelling of two other proteins of higher molecular weights (55 000 and 67 000) with only one of the procedures used (*N*-ethylmaleimide alkylation) suggests the possible involvement of more than one protein in the transport process. Since genetic analysis of the cyclitol-transport system in *K. aerogenes* has not been elucidated, the number of proteins involved is not yet known. The other proteins labelled by *N*-ethylmaleimide must have specific interactions with the cyclitol, since they are protected by high amounts of substrate. An alternative explanation is that *N*-ethylmaleimide-sensitive sites on functional membrane proteins coupled to transport could be masked and become inaccessible to the alkylating agent when cyclitol transport proceeds. Although a precise function cannot be attributed yet to the mol.wt.-55 000 and -67 000 proteins, the results presented in the present paper demonstrate the involvement of an inducible mol.wt.-34 000 carrier protein in the cyclitol-transport system.

The transport system is highly sensitive to diazotized sulphanilic acid. The coupling reaction can be prevented by substrate excess and these properties have been used to label the membrane. From the radioactive pattern of the polyacrylamide-gel electrophoresis it can be seen that only a protein of mol.wt. 33 000 is susceptible to being labelled. Similarly the comparison of induced and non-induced cells led to the conclusion that the same membrane component is labelled.

From the inhibition observed with thiol reagents (Deshusses & Reber, 1974), from the inhibition observed with diazotized sulphanilic acid and protection by substrate, one can assume that the carrier in-

volves thiol groups and aromatic amino acids, probably tyrosine residues, in its binding site.

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