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The organization of hydrogenase in the cytoplasmic membrane of *Escherichia coli*

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The organization of the membrane-bound hydrogenase from *Escherichia coli* was studied by using two membrane-impermeant probes, diazotized [^{125}I]di-iodosulphanilic acid and lactoperoxidase-catalysed radioiodination. The labelling pattern of the enzyme obtained from labelled spheroplasts was compared with that from predominantly inside-out membrane vesicles, after recovery of hydrogenase by immunoprecipitation. The labelling pattern of F_1-ATPase was used as a control for labelling at the cytoplasmic surface throughout these experiments. Hydrogenase (mol.wt. approx. 63000) is transmembranous. Crossed immunoelectrophoresis with anti-(membrane vesicle) immunoglobulins, coupled with successive immunoadsorption of the antiserum with spheroplasts, confirmed the location of hydrogenase at the periplasmic surface. Immunoadsorption with sonicated spheroplasts suggests that the enzyme is also exposed at the cytoplasmic surface. Inside-out vesicles were prepared by agglutination of sonicated spheroplasts, and the results of immunoadsorption using these vesicles confirms the location of hydrogenase at the cytoplasmic surface.

The membrane-bound hydrogenase (EC 1.12.–.–) of *Escherichia coli* is involved in the formate hydrogen-lyase pathway, which converts formate into CO_2 and H_2 (Gray & Gest, 1965) and in the energy-conserving oxidation of H_2 via fumarate reductase (Macy *et al.*, 1976; Bernhard & Gottschalk, 1978; Jones, 1980). The proton-translocating activity of hydrogenase has been established by direct measurement of the stoichiometries (protons translocated per H_2 oxidized) for the oxidation of H_2 by a variety of electron acceptors (Jones, 1979, 1980).

That hydrogenase catalyses proton translocation implies that it is arranged in a transmembranous manner in the cytoplasmic membrane. In the present paper the organization of the enzyme within the membrane is explored by using direct covalent modification with non-membrane-permeant reagents ([^{125}I]DDISA and lactoperoxidase-catalysed incorporation of ^{125}I) and immunoadsorption of anti-(membrane vesicle) immunoglobulins with intact or disrupted spheroplasts. It is concluded that hydrogenase spans the cytoplasmic membrane.

Abbreviations used: [^{125}I]DDISA, diazotized [^{125}I]-di-iodosulphanilic acid; F_1-ATPase, Ca^{2+}- or Mg^{2+}-activated soluble ATPase; SDS, sodium dodecyl sulphate.

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Materials and methods

Growth of Escherichia coli

Escherichia coli (strain A1002) was grown anaerobically in a medium based on that of Cohen & Rickenberg (1956), containing glucose (0.2%, w/v), KNO_3 (1%, w/v), $(NH_4)_6Mo_7O_{24}$ (1μM), K_2SeO_3 (1μM), $MgCl_2$ (1mM), peptone (0.5%, w/v) and isoleucine, valine and methionine (10μg/ml each), adjusted to pH6.4. Growth was at 37°C in 500ml tightly stoppered bottles for anaerobic conditions.

Precultures of strain A1002 were grown aerobically on nutrient broth, then washed once, by centrifugation (7000*g* for 15min) in 50mM-sodium phosphate buffer, pH6.8, before inoculation into the medium described above.

Preparation of spheroplasts and membrane vesicles

Spheroplasts were prepared from sedimented bacteria exactly as described previously (Graham & Boxer, 1981) and were used immediately. Membrane vesicles were prepared from spheroplasts by ultrasonic disruption as described by Graham &

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Boxer (1981) and were stored in liquid N_2 before use.

Labelling with diazotized [^{125}I]di-iodosulphanilic acid

[^{125}I]DDISA was synthesized from sulphanilic acid and Na^{125}I by the procedure used by Helmkamp & Sears (1970). The reagent was finally resuspended in 0.5 M-sucrose/20 mM-sodium phosphate/1 mM-EDTA, pH 7.5 (specific radioactivity approx. 100 Ci/mol) and was used immediately after synthesis.

Spheroplasts (40 mg of protein) and membrane vesicles (9 mg of protein, also in 0.5 M-sucrose/20 mM-sodium phosphate/1 mM-EDTA, pH 7.5) were mixed and [^{125}I]DDISA was added to a final concentration of 1 mM, in a 10 ml volume. After 30 min on ice, labelled spheroplasts and membrane vesicles were separated by differential centrifugation, and membrane vesicles were prepared from the washed labelled spheroplasts (Graham & Boxer, 1981).

The washed labelled vesicle preparations (from spheroplasts and membrane vesicles) were resuspended to the same protein concentration and were made 4% (w/v) in Triton X-100. After 1 h at 4°C, the mixture was centrifuged at 100000 ***g*** for 1 h to remove Triton-insoluble material, and equal volumes of the detergent extracts were used for immunoprecipitation.

Lactoperoxidase-catalysed radioiodination

Spheroplasts (25 mg of protein) and membrane vesicles (7.2 mg of protein) were suspended together at room temperature to a final volume of 0.4 ml in 0.5 M-sucrose/20 mM-sodium phosphate/1 mM-EDTA, pH 7.5, containing 200 μg of lactoperoxidase (Sigma) and 40 nmol of Na^{125}I (specific radioactivity 25 Ci/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.). Five additions each of 1 μmol of H_2O_2 were made at 3 min intervals. Spheroplasts and membrane vesicles were separated by differential centrifugation, and membrane vesicles were prepared from the washed labelled spheroplasts (Graham & Boxer, 1981). Detergent extracts of the washed labelled vesicle preparations (from spheroplasts and membrane vesicles) were prepared as described above.

Preparation of antisera and immunoglobulin fractions

Antiserum to membrane vesicles, de-repressed for hydrogenase synthesis, was prepared as described by Graham *et al.* (1980), and is termed anti-(membrane vesicle) serum.

Monospecific antiserum to hydrogenase was prepared by injecting a rabbit with hydrogenase precipitin arcs as described by Graham *et al.* (1980).

Monospecific antiserum to F_1-ATPase was prepared by using purified F_1-ATPase from *E. coli*, which was a gift from Dr. G. Vogel (University of Tubingen, Federal Republic of Germany).

Monospecific antiserum to nitrate reductase was prepared by injecting a rabbit with nitrate reductase purified by the method of Enoch & Lester (1975).

The immunoglobulin fractions of anti-(membrane vesicle), anti-(hydrogenase) and control sera were prepared essentially as described by Harboe & Ingild (1973), and were stored at −20°C (in one-fifth of the original serum volumes).

Immunoprecipitation

Immunoprecipitation was performed by addition of sufficient of the specified antiserum to precipitate all the antigen present in a Triton-X-100-solubilized extract, as determined by titration. The mixtures were left for 16 h at 4°C, and the immunoprecipitates were collected and washed as described by Werner (1974). The immunoprecipitates were finally resuspended to 50 μl with 50 mM-Tris/HCl, pH 7.5, and disaggregated for SDS/polyacrylamide-gel electrophoresis by the method of Laemmli (1970).

Crossed immunoelectrophoresis

Crossed immunoelectrophoresis of Triton-X-100-solubilized membrane vesicles was performed essentially as described by Smyth *et al.* (1978). Barbital/HCl buffer ($I = 0.04$, pH 8.6) containing 1% (w/v) Triton X-100 was used throughout and was incorporated into all gels.

The Triton X-100 extract used for the crossed-immunoelectrophoresis plates was obtained by dispersing membrane vesicles (18 mg of protein/ml) in 4% (w/v) Triton X-100 for 1 h. The mixture was then ultracentrifuged at 100000 ***g*** for 1 h to remove the Triton-X-100 insoluble material. The Triton X-100 extract was stored in liquid N_2 before use. Electrophoresis of 5 μl samples was performed in the first dimension in 1% (w/v) agarose gels, on 50 mm × 50 mm glass plates, at 4.5 V/cm for 1 h. An agarose strip (10 mm × 50 mm) containing the antigens (electrophoresed in the first dimension) was retained on the glass plate after removal of the rest of the gel, which was replaced with an adjacent gel (40 mm × 50 mm) containing antibody, which fused with the strip containing the antigen. Electrophoresis in the second dimension was then performed at 2 V/cm for 12–18 h.

After electrophoresis, the agarose plates were washed three times in 0.1 M-NaCl and then either stained for activity as described below or stained for protein.

For protein staining the plates were air-dried and stained in 0.5% (w/v) Coomassie Brilliant Blue in 10% (v/v) acetic acid/45% (v/v) methanol for

15 min and destained in 10% (v/v) acetic acid/45% (v/v) methanol.

Activity stains for D-lactate dehydrogenase and NADH dehydrogenase were performed exactly as described by Smyth *et al.* (1978). Hydrogenase precipitin arcs were specifically located by using the H_2-dependent reduction of Methyl Viologen and Benzyl Viologen as described previously (Graham *et al.*, 1980).

Adsorption of anti-(membrane vesicle) immunoglobulins

Spheroplasts (15 mg of protein/ml), prepared as described above, were centrifuged and resuspended in 0.5 M-sucrose/20 mM-sodium barbitone/0.5 mM-EDTA, pH 8.6. Anti-(membrane vesicle) immunoglobulins (0.15 ml) were added to 0–1.0 ml of spheroplasts and the final volumes were adjusted to 1.45 ml with the above buffer. After 1 h at 4°C, the spheroplasts were removed by centrifugation (10000 *g* for 2 min); 1.3 ml portions of the supernatant fractions were mixed with 1.35 ml of 2% (w/v) agarose, containing 2% (w/v) Triton X-100, and were used for crossed immunoelectrophoresis as described above.

Ultrasonically disrupted spheroplasts were prepared from the same spheroplast preparations as above by sonication (Graham & Boxer, 1981) and were used for immunoadsorption as described above.

Polyacrylamide-gel electrophoresis and radioactivity counting

Electrophoresis in the presence of SDS was performed in 10% (w/v) acrylamide separating gels with 3% (w/v) acrylamide stacking gels (Laemmli, 1970). Determinations of molecular weight were made by using the following marker polypeptides: phosphorylase (100000); bovine serum albumin (68000); catalase (60000); immunoglobulin heavy chain (50000); ovalbumin (43000); and lactate dehydrogenase (36000). For accurate determinations of molecular weight, vertical slab-gel electrophoresis was performed, with the acrylamide concentrations described above, although for radioactivity-counting experiments cylindrical gels were used.

After staining for protein, cylindrical gels were sliced uniformly into 1 mm slices with a Mickle gel slicer (Mickle Engineering Co., Gomshall, Surrey, U.K.). The distribution of ^{125}I radioactivity was determined by counting samples directly in a model 300 Packard Auto-Gamma spectrometer.

Assays

Protein was measured by the method of Lowry *et al.* (1951) with bovine serum albumin (Fraction V, BDH) as a standard.

Hydrogenase activity was measured as the hydrogen-dependent reduction of the artificial electron acceptor Benzyl Viologen (Jones, 1979).

Results and discussion

Characterization of anti-(hydrogenase) serum

Anti-(hydrogenase) serum could be used to immunoprecipitate hydrogenase from detergent-solubilized *E. coli* preparations. A titration of Triton X-100-dispersed membrane vesicles with anti-(hydrogenase) serum resulted in the hydrogenase activity (measured as H_2-dependent Benzyl Viologen oxidoreductase) being precipitated by the anti-serum, but not by the pre-immune serum (results not shown). Neither serum inhibited the hydrogenase activity. The H_2:diquat oxidoreductase activity of the enzyme was also unaffected by antiserum or pre-immune serum. Analysis of anti-(hydrogenase) serum by crossed immunoelectrophoresis by using Triton-X-100-solubilized membrane vesicles produced a single protein-stained precipitin arc, which could also be stained for hydrogenase activity before protein staining (Graham *et al.*, 1980).

The polypeptide composition of the immunoprecipitated enzyme was revealed by SDS/polyacrylamide-gel-electrophoretic analysis of material obtained by using a Triton-X-100-solubilized extract of membrane vesicles prepared from [^{35}S]sulphate-grown bacteria. The molecular weight estimated from cylindrical gels was approx. 58000 (Graham *et al.*, 1980); however, when estimated from vertical slab gels it was 63000 ± 2000.

Labelling of membrane-bound hydrogenase with [^{125}I]DDISA

The orientation of hydrogenase within the cytoplasmic membrane was investigated by labelling with [^{125}I]DDISA. The modification of spheroplasts was taken to be equivalent to labelling at the periplasmic surface of the membrane, and that of membrane vesicles, prepared by ultrasonic treatment (which possess predominantly inside-out orientation with respect to the intact cell), to labelling at the cytoplasmic surface of the membrane.

Under the labelling conditions normally used for modification with [^{125}I]DDISA (low reagent concentration), only a small fraction (assumed to be representative) of all potentially active sites on the membrane surfaces is labelled. To ensure that the incorporation of [^{125}I]DDISA at the periplasmic and cytoplasmic surfaces of the membrane were directly comparable, spheroplasts and membrane vesicles were mixed before exposure to the reagent, and were subsequently separated.

The incorporation of label at the cytoplasmic face of the membrane was monitored by the measurement of the extent of modification of F_1-ATPase in

spheroplasts and membrane vesicles. F_1-ATPase, an enzyme which is located exclusively at the cytoplasmic face (Downie *et al.*, 1979), was also recovered by immunoprecipitation from the same iodinated preparations from which hydrogenase was recovered. Fig. 1(*a*) shows that the larger subunits of F_1-ATPase (I and II, mol.wts. 56000 and 52000 respectively) were labelled only in the membrane vesicles, which is consistent with their location and confirms that the iodination had been confined to the membrane surfaces exposed to the bulk phase.

Hydrogenase (mol.wt. 63000), obtained from the same labelled preparations as F_1-ATPase above, was labelled in both spheroplasts and membrane vesicles (Fig. 1*b*). Since the same amounts of membrane protein from spheroplasts and membrane vesicles were used for the solubilization and subsequent immunoprecipitation, then the relative amounts of label incorporated at each surface should be directly comparable. The results suggest that hydrogenase is transmembranous.

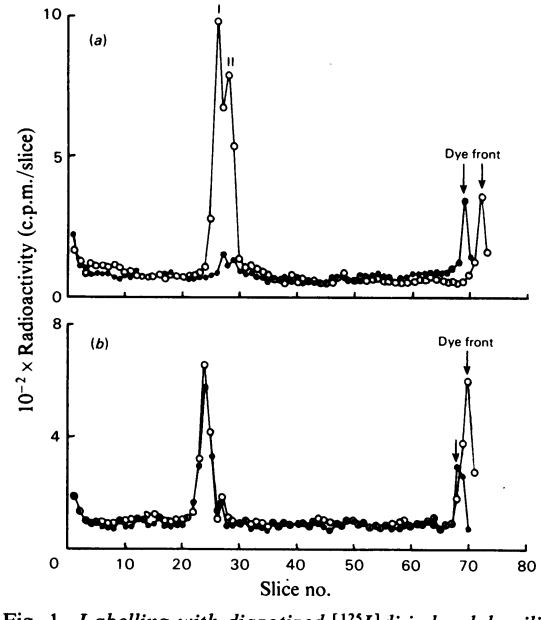

Fig. 1. *Labelling with diazotized [^{125}I]di-iodosulphanilic acid of membrane-bound F_1-ATPase and hydrogenase*
A spheroplast and membrane-vesicle mixture was labelled with [^{125}I]DDISA, then each was separated as described in the Materials and methods section. Membrane vesicles were prepared from the spheroplasts, and both vesicle preparations (at the same protein concentrations) were dispersed separately with Triton X-100. F_1-ATPase (*a*) and hydrogenase (*b*) were immunoprecipitated, with antibodies specific for F_1-ATPase and hydrogenase respectively. Immunoprecipitated material from spheroplasts (●) and from membrane vesicles (○) was analysed by SDS/polyacrylamide-gel electrophoresis. The anode is at the right.

Lactoperoxidase-catalysed radioiodination of membrane-bound hydrogenase

Spheroplasts and membrane vesicles were iodinated as a mixture, to avoid differences in labelling conditions which would occur, since spheroplasts contain higher catalase activities than do membrane vesicles (Graham, 1979). The degree of modification is a function of both the concentration of available sites and the concentration of H_2O_2, a substrate for lactoperoxidase (and catalase). Thus by modifying the mixture and recovering the spheroplasts and membrane vesicles separately by

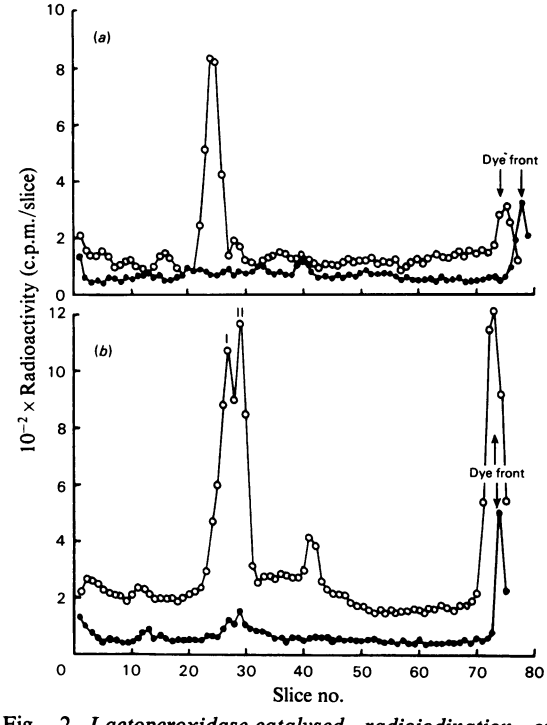

Fig. 2. *Lactoperoxidase-catalysed radioiodination of membrane-bound hydrogenase and F_1-ATPase*
Labelling was performed as described in the Materials and methods section. Spheroplasts and membrane vesicles were mixed and iodinated. After separation, membrane vesicles were prepared from the spheroplasts, and both vesicle preparations (at the same protein concentrations) were dispersed separately with Triton X-100. Hydrogenase (*a*) and F_1-ATPase (*b*) were immunoprecipitated with antibodies specific for hydrogenase and F_1-ATPase respectively. Immunoprecipitated material from spheroplasts (●) and from membrane vesicles (○) was analysed by SDS/polyacrylamide-gel-electrophoresis. The anode is at the right.

differential centrifugation, the extent of iodination of the two preparations could be compared directly (Graham & Boxer, 1981).

The incorporation of ^{125}I into hydrogenase from modified spheroplasts and membrane vesicles is shown in Fig. 2(*a*). The enzyme is not iodinated in spheroplasts, but is labelled in membrane vesicles, and so is located, at least in part, on the cytoplasmic surface of the membrane.

Fig. 2(*b*) shows that the two larger subunits of F_1-ATPase (I and II) were iodinated only in the membrane vesicles, which is consistent with its known location at the cytoplasmic surface. The labelling patterns (Figs. 2*a* and 2*b*) show that there are peaks of ^{125}I radioactivity at the dye front. These can be partially decreased by lipid extraction, and therefore correspond to iodinated lipid or glycolipid.

Nitrate reductase and formate dehydrogenase were recovered by immunoprecipitation, with their respective antisera, from the same labelled preparations as F_1-ATPase and hydrogenase (results not shown). The results are in complete agreement with the results obtained from studies using *E. coli* grown anaerobically on nitrate (Graham & Boxer, 1978, 1980*a*,*b*, 1981). It is worth noting that hydrogenase could not be labelled by lactoperoxidase-catalysed radioiodination of spheroplasts under conditions when the α-subunit of formate dehydrogenase (mol.wt. 110000) could be labelled. This suggests that the lack of labelling of hydrogenase cannot be due to outer-membrane fragments remaining bound to the inner membrane causing the periplasmic surface to be inaccessible.

Antigenic architecture of E. coli membranes established by crossed immunoelectrophoresis

In order to assess the extent to which different specific immunogens are expressed on the membrane surface, anti-(membrane vesicle) immunoglobulins were adsorbed with increasing quantities of spheroplasts or ultrasonically disrupted spheroplasts. Antibodies directed against membrane immunogens present on the outer surface of the cytoplasmic membrane (or which are transmembranous) should be adsorbed with spheroplasts. Antibodies to membrane immunogens present on the inner surface (or which are transmembranous) should be adsorbed with ultrasonically disrupted spheroplasts.

Crossed immunoelectrophoresis of Triton-X-100-solubilized membrane vesicles (prepared from *E. coli* de-repressed for hydrogenase synthesis) against antiserum raised to these membrane vesicles produced, as expected, a large number of protein-stained precipitin arcs (Fig. 3*a*), as reported previously (Graham *et al.*, 1980). Three precipitin arcs in this pattern were identified by zymogram staining: hydrogenase, NADH dehydrogenase and D-lactate dehydrogenase (Figs. 3*b*–3*d* respectively). Nitrate reductase was identified by the use of monospecific antiserum to nitrate reductase being incorporated into the agarose in place of anti-(membrane vesicle) immunoglobulins (Fig. 3*e*). The nitrate reductase arc was confirmed by activity staining by the method of Lund & DeMoss (1976). ATPase was identified by the use of monospecific antiserum to F_1-ATPase being incorporated into the plate in addition to anti-(membrane vesicle) immunoglobulins (Fig. 3*f*). The peak area of only the ATPase arc was decreased by this addition.

NADH dehydrogenase, D-lactate dehydrogenase and ATPase have been shown to be located exclusively at the cytoplasmic face in *E. coli* by immunoadsorption experiments (Owen & Kaback, 1978), and therefore should be markers for the accessibility of this surface in these immunoadsorption experiments.

The immunoplates in Fig. 4 represent a series of typical spheroplast adsorption experiments in which the plates were stained for D-lactate dehydrogenase activity to aid observation and subsequently stained for protein. It is visually obvious that the peak areas subtended by immunoprecipitates corresponding to D-lactate dehydrogenase (antigen 1), nitrate reductase (antigen 2), ATPase (3) and NADH dehydrogenase (4) do not increase significantly (i.e. antibody is not adsorbed) when spheroplasts are used for immunoadsorption. Hydrogenase (antigen 5), as well as some other unidentified precipitin arcs, shows a significant increase in peak area when anti-(membrane vesicle) immunoglobulins are immunoadsorbed with spheroplasts. Since the antibodies to hydrogenase are adsorbed by spheroplasts, this demonstrates that hydrogenase is present, at least in part, on the periplasmic surface of the cytoplasmic membrane.

Similar experiments performed with ultrasonically disrupted spheroplasts resulted in all precipitin arcs being adsorbed progressively; indeed, when 0.3 ml of sonicated spheroplasts was used no precipitin arcs were observed on the plates.

Data from these experiments are represented graphically in Fig. 5, where the reciprocal of the area subtended by the immunoprecipitate is plotted as a function of the quantity of intact or ultrasonically disrupted spheroplasts used during immunoadsorption (*v*). This method was first applied for *E. coli* membrane vesicles by Owen & Kaback (1978).

It is clear that the membrane-bound immunogens fall into two categories: (i) those typified by D-lactate dehydrogenase, ATPase and nitrate reductase, antibodies to which are not adsorbed to a significant extent with spheroplasts relative to ultrasonically disrupted spheroplasts (NADH dehydrogenase also falls into this category; however, the results are not shown in Fig. 5); (ii) those typified by hydrogenase,

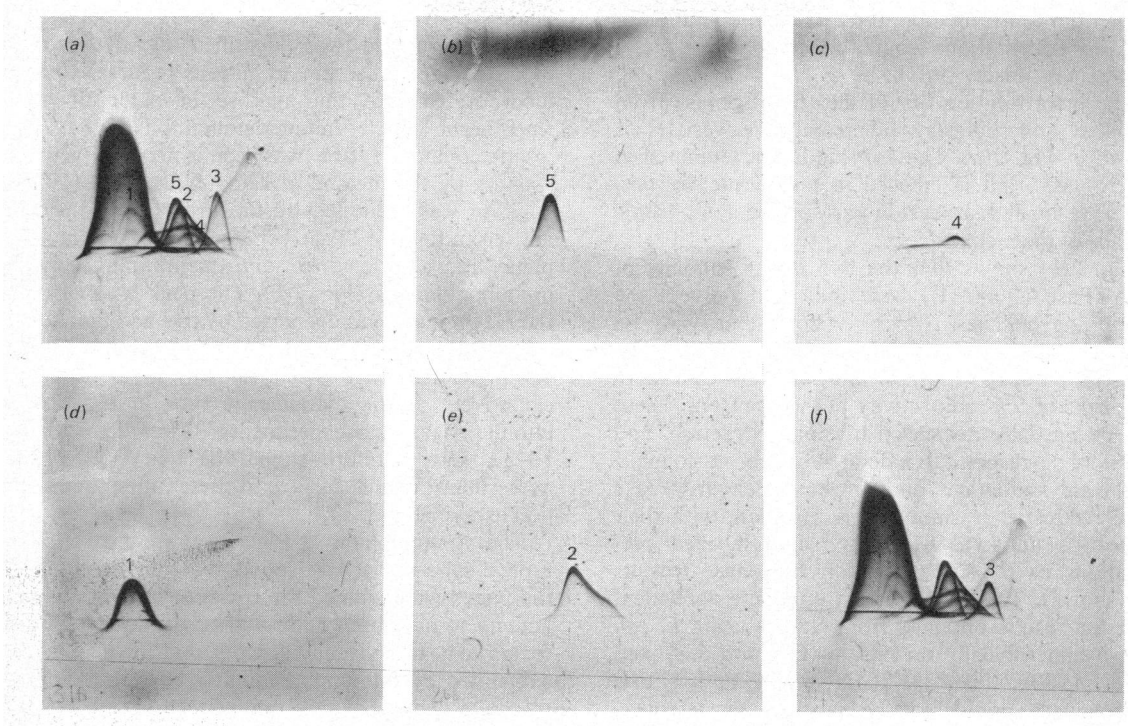

Fig. 3. ***Characterization of immunoprecipitates in the crossed-immunoelectrophoresis reference pattern for membrane vesicles of E. coli***

Crossed immunoelectrophoresis was performed as described in the Materials and methods section. Samples (5 μl) of Triton X-100-dispersed membrane vesicles were electrophoresed against anti-(membrane vesicle) immunoglobulins. After electrophoresis, immunoplates were stained for either protein (*a*, *e* and *f*) or activity. Plates similar to the protein-stained one in (*a*) were activity stained for hydrogenase (*b*), NADH dehydrogenase (*c*) and D-lactate dehydrogenase (*d*). Plate (*e*) was obtained by electrophoresis of the Triton X-100 extract against monospecific anti-(nitrate reductase) serum, followed by protein staining. Plate (*f*) shows the protein-stained plate obtained by electrophoresis of the Triton X-100 extract against a mixture of anti-(membrane vesicle) immunoglobulins and specific anti-(F_1-ATPase) serum.

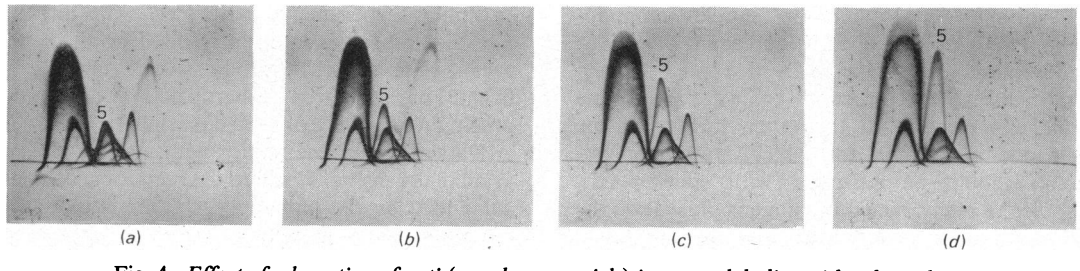

Fig. 4. *Effect of adsorption of anti-(membrane vesicle) immunoglobulins with spheroplasts*

Anti-(membrane vesicle) immunoglobulins were adsorbed with 0 ml (*a*), 0.2 ml (*b*), 0.5 ml (*c*) and 1.0 ml (*d*) of spheroplasts as described in the Materials and methods section. After removal of spheroplasts by centrifugation, the immunoglobulins were incorporated into agarose gels. Immunoplates were stained for D-lactate dehydrogenase activity and then for protein. The hydrogenase precipitin arc is shown (5).

antibodies to which are removed by spheroplasts and also to a greater extent by ultrasonically disrupted spheroplasts. Although the relative proportion of right-side and inside-out vesicles is not known for this preparation of sonicated spheroplasts, the results would suggest that there are more hydrogenase antigenic sites available after sonication and that the enzyme is therefore transmem-

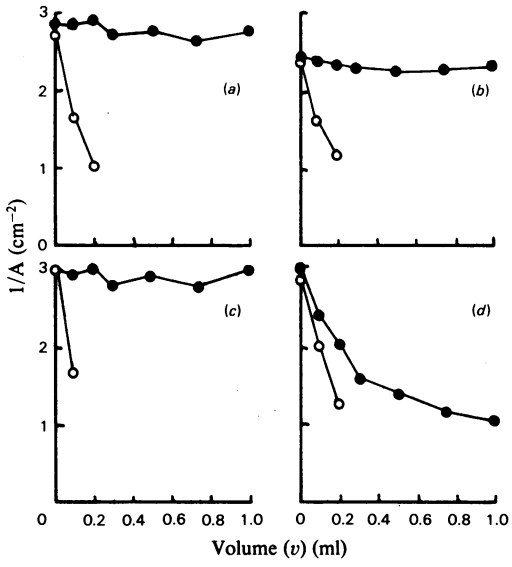

Fig. 5. *Effect of progressive adsorption of anti-(membrane vesicle) immunoglobulins with spheroplasts and disrupted spheroplasts on the peak areas of immunoprecipitates*

The effect of immunoadosrption on the following peak areas is shown: (*a*) D-lactate dehydrogenase (antigen no. 1), (*b*) nitrate reductase (antigen no. 2), (*c*) ATPase (antigen no. 3), and (*d*) hydrogenase (antigen no. 5). Anti-(membrane vesicle) immunoglobulin was adsorbed with increasing volumes (*v*) of spheroplasts (●) as described in the Materials and methods section. Similar experiments were conducted with the same spheroplasts which had been disrupted by sonication (○). After immunoadsorption, the immunoglobulins were incorporated into agarose gels and analysed against a Triton X-100 extract of membrane vesicles by crossed immunoelectrophoresis. The peak areas (A) were estimated by photographically enlarging (×5) images of each immunoplate and excising and weighing five copies of each peak. The average weights were then computed.

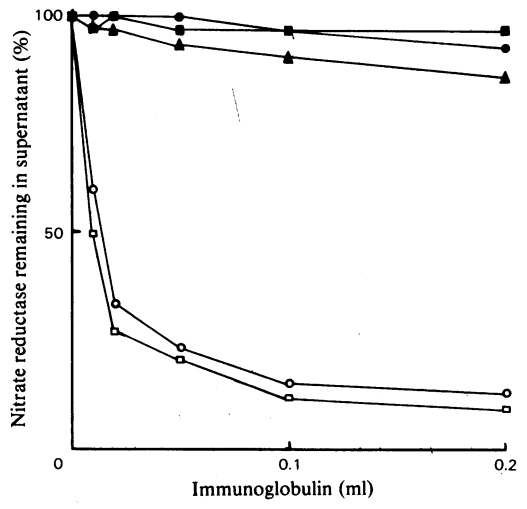

Fig. 6. *Agglutination of membrane vesicles with anti-(F_1-ATPase) and anti-(nitrate reductase) immunoglobulins*

Ultrasonically disrupted spheroplasts (0.1 ml, 1.5 mg of protein) were incubated with the specified amount of control, anti-(F_1-ATPase) or anti-(nitrate reductase) immunoglobulins (in a final volume of 1 ml) for 16 h at 4°C. The agglutinated vesicles were removed by centrifugation (480 *g* for 5 min). The supernatant fractions (non-agglutinated vesicles) were assayed for nitrate reductase activity. ▲, Control immunoglobulins after centrifugation; ■, anti-(F_1-ATPase) before centrifugation; □, anti-(F_1-ATPase) after centrifugation; ●, anti-(nitrate reductase) before centrifugation; ○, anti-(nitrate reductase) after centrifugation.

branous. However, it is possible that in the spheroplast preparation not all the surface immunogens are available to the bulk phase, owing to the presence of outer cell wall remaining on the spheroplasts, and that ultrasonication removes these outer-membrane fragments to expose all the antigenic sites (i.e. hydrogenase in particular) at this surface.

Heterogeneity of orientation of ultrasonically disrupted spheroplasts

It has been reported that membrane vesicles from *E. coli* prepared by ultrasonic disruption are largely inverted with respect to whole cells (Hare *et al.*, 1974; Futai, 1974). The heterogeneity of the ultrasonically disrupted spheroplasts used in the experiments above was estimated by a method similar to that used by Hare *et al.* (1974). This is important, since it should help in the interpretation of the immunoadsorption experiments.

Antisera to both F_1-ATPase (Graham, 1979) and nitrate reductase (Graham & Boxer, 1978) have been shown, by immunofluorescence studies, to bind to membrane vesicles, but not to spheroplasts. This knowledge was exploited to fractionate membrane vesicles into populations of defined and opposite orientation. Both antisera are monospecific for their respective antigens (as shown by crossed immunoelectrophoresis with detergent extracts of membrane vesicles).

Ultrasonically disrupted spheroplasts, from the same preparation used for the immunoadsorption experiments above, were challenged with anti-(F_1-ATPase) or anti-(nitrate reductase) immunoglobulin fraction, as shown in Fig. 6. The nitrate reductase

activity was used as a marker for the membranes, and about 90% of the vesicles could be agglutinated. The agglutinated vesicles bind to the antisera, and so have at least part of their cytoplasmic surface exposed to the bulk phase.

The agglutinated and 'total' membrane-vesicle preparations were used for immunoadsorption studies against anti-(membrane vesicle) immunoglobulins (results not shown). Immunoadsorption of anti-(membrane vesicle) immunoglobulins with a preparation of 'total' ultrasonically disrupted spheroplasts (in comparison with a control plate which had no immunoadsorption) showed that most of the precipitin arcs, including D-lactate dehydrogenase, nitrate reductase, ATPase and hydrogenase, increased in area. This suggests that these proteins are located, at least in part, at the cytoplasmic surface.

When ultrasonically disrupted spheroplasts, which had been immunologically subfractionated by using antibodies to F_1-ATPase, were used to immunoadsorb anti-(membrane vesicle) immunoglobulins, the peak areas of D-lactate dehydrogenase, nitrate reductase and hydrogenase increased (results not shown). This is consistent with these proteins being exposed in the agglutinated membrane vesicles and hence located at the cytoplasmic surface. The peak area of ATPase did not increase because antibodies to F_1-ATPase, used to agglutinate the vesicles, must still be bound to the ATPase on the membrane vesicle surface.

Concluding remarks

Concordant results regarding the topography of hydrogenase in the cytoplasmic membrane of *E. coli* have been obtained with immunoadsorption studies and with more conventional vectorial chemical labelling probes (lactoperoxidase-catalysed radioiodination and [^{125}I]DDISA labelling). Because of the inherent limitation of each method it is useful to employ all three techniques, thereby obtaining the most reliable picture of the topography of hydrogenase.

The interpretation of the localization studies was facilitated by the use of markers for the cytoplasmic surface (F_1-ATPase for chemical labelling studies and ATPase, D-lactate dehydrogenase and NADH dehydrogenase for immunoadsorption studies). Distinct locations were obtained for these markers with intact spheroplasts and disrupted spheroplasts (membrane vesicles), thus demonstrating the intactness of the spheroplasts and the insignificant membrane permeation of the labelling probes used. Unfortunately no protein located exclusively at the periplasmic surface of the membrane has been identified at present. Such a component would serve as a valuable control for the accessibility of this surface to the labelling probes.

The method of labelling mixtures of spheroplasts and membrane vesicles for the establishment of the location of a membrane component has been used previously in studies of the topography of formate dehydrogenase of *E. coli* (Graham & Boxer, 1981) and proved most useful in the present study. Without the knowledge that the extent of modification is similar in both preparations of opposite orientation, it is difficult to prove the transmembranous nature of a membrane component by direct coupling, unless peptide 'mapping' is also undertaken. Lactoperoxidase-catalysed radioiodination resulted in the hydrogenase being labelled only at the cytoplasmic surface, and so such complications of interpretation do not arise. However, labelling mixtures was important in the work with [^{125}I]DDISA, since hydrogenase could be labelled at both the periplasmic and cytoplasmic surfaces.

Hydrogenase is structurally and functionally transmembranous, and consists of only one polypeptide, and therefore is an attractive candidate for the investigation of the mechanism of respiratory proton translocation, and of the synthesis and assembly of a bacterial membrane-bound protein.

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