The Protein Composition of the Cytoplasmic Membrane of Aerobically and Anaerobically Grown *Escherichia coli*

Richard G. F. Visser,[1] Klaas J. Hellingwerf,[1] and Wil N. Konings[1,2]

Abstract

The protein composition of the cytoplasmic membranes of *Escherichia coli,* grown aerobically and anaerobically on a glucose minimal medium at pH 7.0, were analyzed by crossed immunoelectrophoresis. Qualitative differences are limited to only two proteins: nitrate reductase (E.C. 1.7.99.4) is absent under aerobic growth conditions, whereas an unidentified protein, with a molecular weight of 81,500 and located at the inner side of the cytoplasmic membrane, is synthesized only in the presence of oxygen. Quantitative differences are observed for many proteins: the ratio of the amount of a specific protein present in cells grown anaerobically and aerobically was, for four proteins, between 0.3 and 1; for 25 proteins, between 1 and 3; and for five proteins, larger than 5.

Key Words: *Escherichia coli;* crossed immunoelectrophoresis; cytoplasmic membrane proteins; aerobic growth; anaerobic growth.

Introduction

Escherichia coli, a facultative (an)aerobic and heterotrophic bacterium, can adapt rapidly to changes in the environment. Aerobically, it can grow on many sugars and organic acids as carbon, energy, and electron donor, and oxygen as terminal electron acceptor. Under anaerobic conditions, external electron acceptors such as fumarate or nitrate can replace oxygen. When all external electron acceptors are exhausted, fermentative growth is still possible with a number of sugars as carbon and energy source (Stanier *et al.,* 1976; Haddock and Jones, 1977). The energy necessary for growth is then generated by

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[1]Department of Microbiology, Biological Center, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands.
[2]To whom correspondence should be addressed.

electron transfer to fumarate or nitrate, substrate level phosphorylation, and end-product efflux (ten Brink and Konings, 1980; Konings and Boonstra, 1977).

A number of years ago a considerable amount of research was directed toward elucidation of the difference between *E. coli* cells grown aerobically and anaerobically and the regulation thereof (Gray *et al.,* 1966a,b; Wimpenny, 1969; Wimpenny and Cole, 1967; Wimpenny and Necklen, 1971; Wimpenny and Firth, 1972). These studies demonstrated that the enzymes of the tricarboxylic acid cycle, the cytochrome content of the cytoplasmic membrane, and the intracellular NAD concentration are the main targets of regulation during the aerobic/anaerobic transition.

Studies on the comparison of the composition of the proteins of the cytoplasmic membrane of *E. coli* during the aerobic/anaerobic transition are surprisingly few. Spencer and Guest (1974) concluded that the composition of membrane proteins between the two growth conditions differs considerably, whereas Schnaitman (1970), who examined the complete cell envelope, reported only minor differences. In both reports the one-dimensional SDS–PAA[3] gel electrophoresis technique was used for the analysis.

Since these studies, two-dimensional electrophoresis techniques have been developed such as the combination of isoelectrofocusing and SDS–PAA gel electrophoresis (O'Farrel, 1975) and crossed immunoelectrophoresis (CIE) (Owen and Kaback, 1979; van der Plas *et al.,* 1983). These techniques have a considerably higher resolution and capacity to allow recognition of specific proteins in the gels. Therefore we decided to use CIE to analyze the protein composition of the cytoplasmic membrane of *E. coli* during an aerobic/anaerobic transition to resolve the (apparent) contradiction.

Materials and Methods

Cell Growth and Preparation of Membrane Vesicles

E. coli ML308-225 (*lac i⁻z⁻y⁺a*) was grown aerobically and anaerobically at 37°C in a stirred 4-liter fermenter (Harder *et al.,* 1974) containing 3 liters mineral medium A (Davis and Mingioli, 1950), and supplemented with 0.1% (v/v) trace-element solution (Gibson *et al.,* 1979) when cells were grown anaerobically. During growth the pH was controlled at 7.0 by automatic adjustment with 1 N NaOH. Aerobically, cells were grown at 50% air saturation (DOT 77.5 mm Hg). The dissolved oxygen concentration was measured with a Mackereth-type oxygen probe of home-built design (Mei-

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[3]Abbreviations: SDS–PAA, sodium dodecyl sulfate–polyacrylamide; CIE, crossed immunoelectrophoresis.

berg *et al.,* 1980). Anaerobically, cells were grown under White Spot Nitrogen. Glucose 0.5% (w/v) was used as a carbon source in all experiments.

Membrane vesicles were prepared according to Kaback (1971) or to Konings and Kaback (1973) when cells were grown aerobically or anaerobically, respectively, and were stored in liquid nitrogen. They are referred to as glucose/O_2 and glucose/N_2 vesicles, respectively.

Preparation of Triton X-100 Extracts for Crossed Immunoelectrophoretic Analysis

Membrane vesicles were suspended, after centrifugation (48,000 × *g;* 4°C, 30 min), in 50 mM Tris-HCl, pH 8.6, 5 mM EDTA, and 9% (w/v) Triton X-100 at a protein concentration of 10 mg/ml. After incubation for 1 h at 20°C the suspension was centrifuged (48,000 × *g,* 4°C, 60 min) and the supernatant was stored in 50-μl portions in liquid nitrogen.

Isolation of Antibodies

Antibodies against glucose/nitrate, glycerol/fumarate, and glucose/O_2 membrane vesicles were raised and isolated essentially as described before (van der Plas *et al.,* 1983; Elferink *et al.,* 1979; Joustra and Lundgren, 1969). Aliquots of the three antibody preparations were pooled in a 1:1:1 volume ratio, concentrated (van der Plas *et al.,* 1983) to ~100 mg/ml, and stored at −20°C. These antibodies are referred to as the antibody pool.

Crossed Immunoelectrophoresis

Crossed immunoelectrophoresis was carried out as described previously (Elferink *et al.,* 1979). In all figures the anode is at the left and top of the figure. Zymogram staining techniques were described by van der Plas *et al.* (1983), except for NADPH-dehydrogenase, which was stained as NADH-dehydrogenase, but with NADPH as the substrate, and *N,N,N,N*-tetramethyl-1,4-phenylenediamine dihydrochloride (TMPD) oxidase activity, which was assayed according to Krantz and Gennis (1982).

Cytochrome Spectra

Oxidation/reduction difference spectra of cytochromes were recorded using an Aminco DW-2 UV-Vis spectrophotometer (American Instruments Company, Silver Springs, Maryland) at room temperature. Spectra of oxidized cytochromes were recorded with membrane vesicles in 1 ml 50 mM potassium phosphate buffer, pH 6.6, after addition of Triton X-100 [5% (w/v) final concentration]. The cytochromes were reduced with a few grains of solid sodium dithionite. Concentrations were calculated on the basis of the extinc-

tion coefficients given by Reid and Ingledew (1980): 12 and 5.3 mM^{-1} · cm^{-1} for cytochrome b and d, respectively.

Miscellaneous

Routine procedures were used for adsorption experiments (van der Plas *et al.*, 1983; Elferink *et al.*, 1979), protein determination (Lowry *et al.*, 1951;
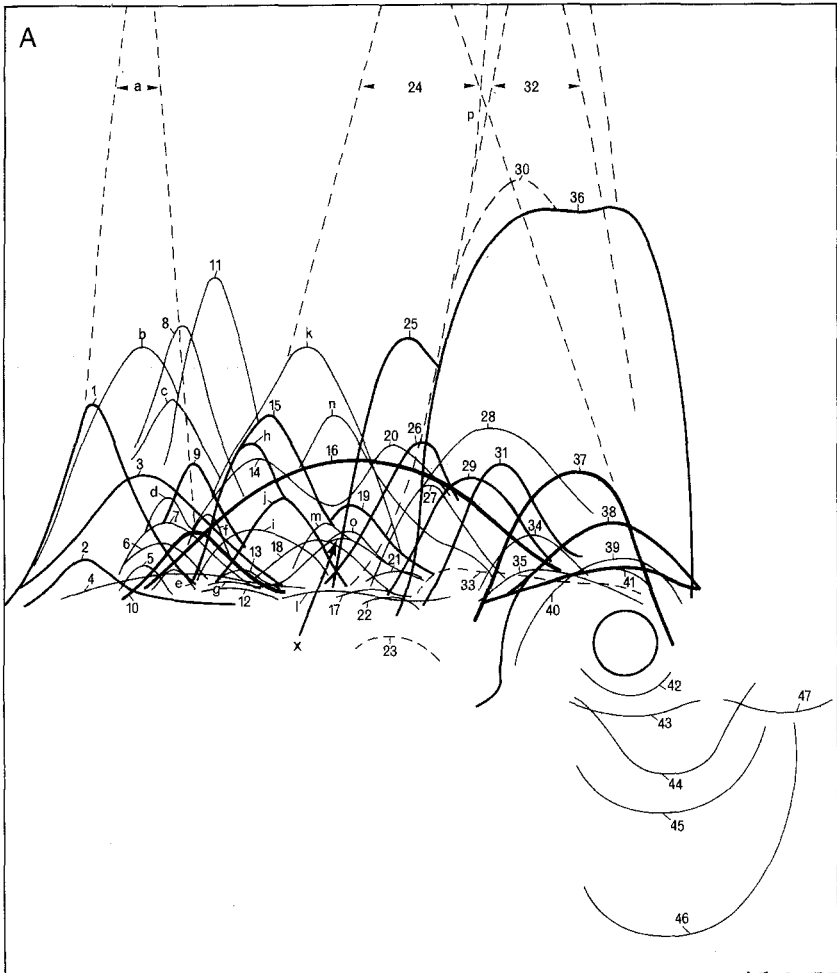

Fig. 1. Schematic representation of crossed immunoelectrophoregrams of glucose/O$_2$ (A) and glucose/N$_2$ (B) in cytoplasmic membrane vesicles. The thickness of the lines reflects the intensity of the Coomassie brilliant blue stain, observed for the various immunoprecipitates. Dotted lines denote precipitates detectable only with more than 17 mg of the antibody pool in the second-dimension gel. Corresponding immunoprecipitates are numbered equally in A and B; the others are lettered.

Dully and Grieve, 1975), assays of the enzyme activity of NADH-dehydrogenase (Enoch and Lester, 1972), D-lactate dehydrogenase (Snoswell, 1963) and succinate dehydrogenase (Ackrell *et al.,* 1978), polarographic measurements of the rate of oxygen consumption at 25°C (Matin and Konings, 1973), and for SDS–PAA gel electrophoresis (Laemmli and Faure, 1973). After staining with Coomassie brilliant blue, the gels were analyzed with a Yoyce-Loebl double-beam recording microdensitometer MK IIIC (Electronhouse, Gateshead-on-Tyne II, England).

Results

CIE Reference Patterns

Membrane vesicles were prepared from cells grown on glucose minimal medium under aerobic and anaerobic conditions, and after solubilization the
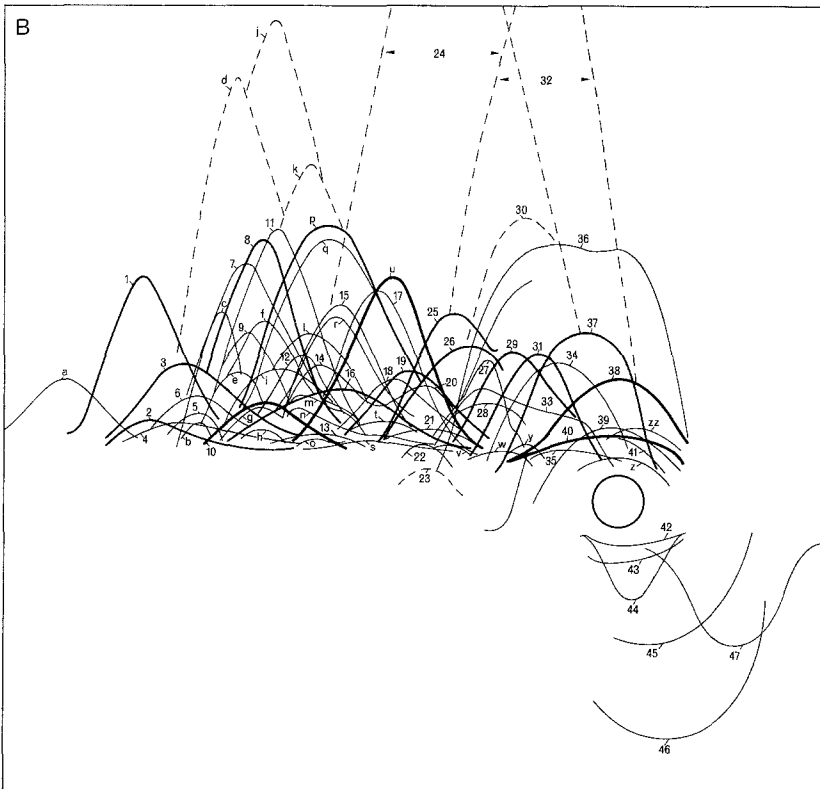
Fig. 1. Continued.

Table I. Characterization of the Precipitation Lines from Crossed Immunoelectrophoregrams from Glucose/O_2 and Glucose/N_2 Vesicles

Enzyme activity	Line number (letter)[a]
6-Phosphogluconate dehydrogenase	5
Heme-associated peroxidation ⎫	
Cytochrome oxidase ⎬	7
TMPD oxidation ⎭	
ATPase ·	10
Succinate oxidation[b]	12, 27, *v*
NADH dehydrogenase	13, *g*
NADPH dehydrogenase	14, 20
Hydrogenase	17
Formate dehydrogenase	21
Malate dehydrogenase	22, 23
D-lactate dehydrogenase	29
α-Glycerol-phosphate dehydrogenase	33
Nitrate reductase	*s*

[a]The numbered lines are present in immunoplates with either type of vesicle.
[b]Lines 12 and 27 are due to fumarate reductase and line *v* to succinate dehydrogenase (van der Plas *et al.*, 1983).

membrane proteins were subjected to CIE. A series of plates was run with membrane protein and pool antibodies, ranging from 40–55 μg and 2.2–44 mg, respectively. From these plates reference patterns were composed (Fig. 1). The reference patterns show that the majority of proteins (47) are present both in glucose/O_2 and in anaerobic glucose/N_2 vesicles, although the relative amounts of the respective proteins vary (see also Table II). In addition, some (17 for aerobic and 27 for anaerobic vesicles) proteins appear to be present; these occur, in this concentration range, in one of the two preparations only (these precipitation lines have been lettered). With the zymogram-staining technique, 17 of the 91 different precipitation lines have so far been identified (Table I).

Table II. The Relation Between the Amount of a Particular Enzyme and Its Specific Activity

Enzyme	Relative[a] amount of protein[b]	Relative oxidase activity[c]	Relative specific dehydrogenase activity
D-lactate dehydrogenase	0.50	2.5	1.1
NADH dehydrogenase	0.57	6.1	1.5
Succinate dehydrogenase	0.39	5.4	6.0[d]
Formate dehydrogenase	0.57	0.8	n.d.[e]

[a]Relative is used to define the ratio of the amount present in glucose/O_2 over the amount present in glucose/N_2 membrane vesicles.
[b]Measured from the area of the particular precipitation line in immunoplates.
[c]Measured polarographically as indicated in Materials and Methods.
[d]Some interference by fumarate reductase may occur in this assay (Van der Plas *et al.*, 1983).
[e]n.d.: not determined.

Quantitative Differences Between Glucose/O_2 and Glucose/N_2 Vesicles

a. From Redox Spectra. From the dithionite-induced redox-difference spectra, the cytochrome composition and content were measured in membrane vesicles from aerobic and anaerobic cells (Fig. 2). The peaks at 562, 597, and 630 nm correspond to cytochrome(s) of the *b*, a_1, and *d* type, respectively (Gray *et al.,* 1966a). Cytochrome a_1 is apparently present in glucose/O_2 vesicles in very limited amounts only. From the extinction coefficients given by Reid and Ingledew (1980), the cytochrome *b* content of glucose/O_2 and glucose/N_2 vesicles is 623 and 296 nmol per gram protein, whereas the corresponding numbers for cytochrome d are 74 and 204 nmol.

b. From Immunoplates. Crossed immunoelectrophoresis has the advantage that the surface areas of the precipitation lines are directly proportional to the amount of antigen (membrane protein) used (Owen and Kaback, 1979). We analyzed the relative amounts of membrane proteins present in glucose/N_2 and glucose/O_2 vesicles of 34 different precipitation lines. Most proteins are present in comparable quantities (25 in glucose/O_2 and glucose/N_2 vesicles). The ratio of the quantity present in glucose/N_2 over glucose/O_2 vesicles is, for 25 proteins, between 1 and 3, and for four proteins, between 0.3 and 1. Only for five proteins was this ratio larger than 5, and for two of these proteins (hydrogenase, line 17 and succinate oxidase, line 12) even higher than 25.

Table II demonstrates that these data on the relative amounts of a particular enzyme cannot be directly translated into enzyme activities. Three different dehydrogenases are present in lower quantities under aerobic conditions. Yet the specific activity is considerably higher (especially for NADH- and succinate dehydrogenase); however, the ratio of specific activities again depends on the particular assay used (Table II). For formate dehydrogenase there is reasonable agreement between the two ratios.
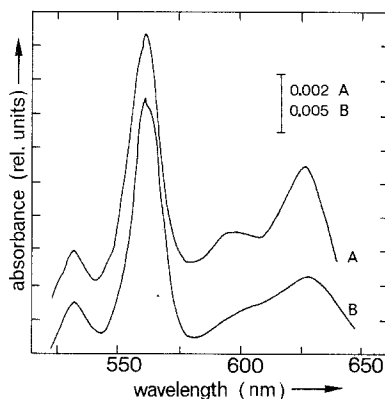
Fig. 2. Redox difference spectra of glucose/O_2 (B) and glucose/N_2 (A) membrane vesicles. The protein concentration was 3.5 mg/ml for A and 4 mg/ml for B.

α-Glycerol-phosphate oxidase activity was very low, both in glucose/O_2 and in glucose/N_2 vesicles.

Qualitative Differences Between Glucose/O_2 and Glucose/N_2 Vesicles

To detect components specifically present either during growth on glucose/O_2 or on glucose/N_2, antibody-pool immunoglobulins (8.8 mg) were absorbed with increasing amounts of solubilized glucose/O_2 membrane vesicles (180–2500 μg protein) or solubilized glucose/N_2 membrane vesicles (600–3600 μg protein). Subsequent CIE with the absorbed antibodies demonstrated (Fig. 3) that one specific component is formed during each growth condition. Precipitation line s (= nitrate reductase, see Table I) is present only in glucose/N_2 vesicles and precipitation line X only in glucose/O_2 vesicles. The properties of the protein that gives rise to precipitation line X (protein X) have been investigated with SDS–PAA gel electrophoresis. Analysis of the supernatant obtained after titration of solubilized glucose/O_2 membrane vesicles with increasing amounts of antibodies (Fig. 4) showed an increased precipitation of all proteins except for protein X (the other bands in the gel (g) are caused by nonprecipitated antibodies). In membrane proteins from glucose/N_2 vesicles this component is missing (gel a in Fig. 4). Figure 4B shows the relative increase of protein X over an arbitrary protein (protein I) upon addition of increasing amounts of antibody.

The molecular weight of protein X is 81,500. Its localization, with respect to the cytoplasmic membrane, was investigated with an absorption experiment (Owen and Kaback, 1979; van der Plas *et al.*, 1983; Elferink *et al.*, 1979). Table III indicates that protein X has the same localization as two proteins that have been taken as a reference for the matrix (inner) side of the cytoplasmic membrane; D-lactate dehydrogenase and NADH-dehydrogenase.

Since protein X is specific for aerobic growth conditions, we investigated the relative amount of this protein as a function of the oxygen concentration during growth (Fig. 5). It turned out that the relative amount of this protein (here expressed as mm^2 of the peak of protein X in a densitometric scan of an

Table III. The Localization of Protein X with Respect to the Cytoplasmic Membrane

| Antigen	Precipitation line[a]	Percent exposed at outer surface[b]
NADH dehydrogenase	13	15 (10)
D-Lactate dehydrogenase	29	26(3)
Protein X	x	23(5)

[a] For nomenclature, see Fig. 1.
[b] The values are the mean of three to four independent absorption experiments. The values in parentheses indicate the magnitude of the standard deviation.
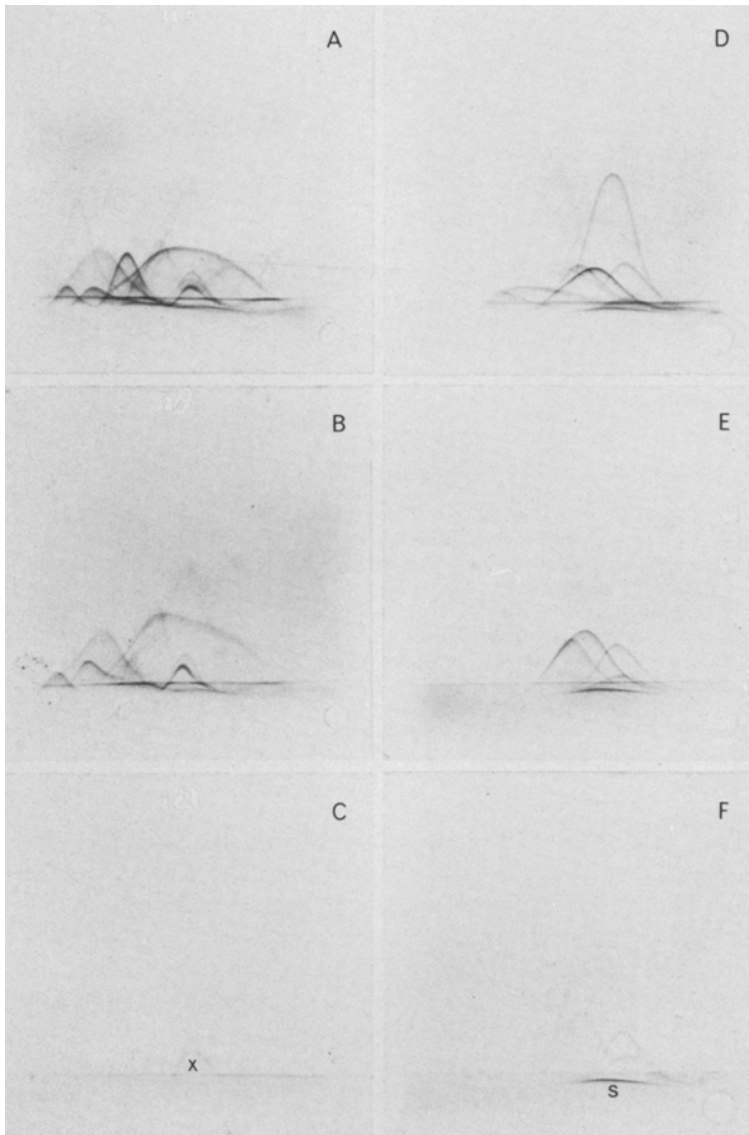
Fig. 3. Absorption of antibody pool immunoglobulins with increasing amounts of glucose/N_2 (A–C) and glucose/O_2 (D–F) membrane vesicles. A–C: 36 μg glucose/O_2 membrane-vesicle protein was brought into the application spot for electrophoresis in the first dimension and run in the second dimension against 8.8 mg antibodies, absorbed with 180 (A), 625 (B), or 2500 μg (C) membrane-vesicle protein. D–F: 40 μg protein was used in the first dimension and the absorption of 8.8 mg antibodies was with 600 (D), 1800 (E), or 3600 μg membrane-vesicle protein. The precipitation lines *x* and *s* are indicated in Fig. 1.
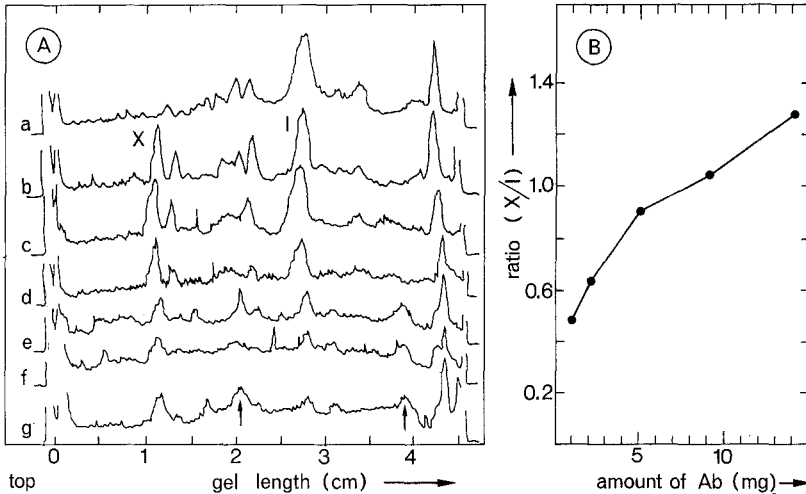
Fig. 4. SDS–PAA gel electrophoresis of the membrane protein (X), specific for aerobic growth. A. Densitometric scans of SDS–PAA gels of: (a) 55 μg glucose/N_2 membrane vesicle protein; (b) 48 μg glucose/O_2 membrane-vesicle protein. (c–g) Progressive adsorption of glucose/O_2 membrane vesicles, with a 1:1 (v/v) mixture of antiglucose/nitrate and antiglycerol/fumarate antibodies. The amount of antibody added to 150 μl glucose/O_2 vesicles (total 1.2 mg protein) was 1 mg (c), 2 mg (d), 5 mg (e), 9 mg (f), and 14 mg (g). After adsorption the mixture was centrifuged (48,000 × g, 4°C, 30 min) and 50 μl of the supernatant was electrophoresed in an SDS–PAA gel. X represents protein X and I one of the major proteins from glucose/N_2 vesicles. The arrows indicate the major peaks of the nonprecipitated antibodies. B. Ratio of the peak areas of the densitometric scans of proteins X and I with increasing amounts of absorbed protein. Each point is taken from Fig. 4A, traces c–g.

SDS–PAA gel to which 100 μg protein of a particular gel sample had been applied) increased with increasing oxygen concentrations. A half-maximal amount of protein X is expressed at approximately 30% air saturation.

In addition to the enzymes positively identified via zymogram staining (Table I), a number of enzymes turned out to be absent in routine staining techniques: formate dehydrogenase "H," acid phosphatase, β-galactosidase, catalase, glycine reductase, proline dehydrogenase, peptidase, disaccharidase, alanine dehydrogenase, L-lactate dehydrogenase, dihydroorotate dehydrogenase, and phosphate-releasing enzyme. Also lipopolysaccharide was not detected in these preparations via a specific staining.

Discussion

The number of detectable immunoprecipitates in membrane vesicles from glucose/O_2-and glucose/N_2-grown cells and the number of basic proteins migrating toward the cathode ($pI > 8.6$) closely correspond to those
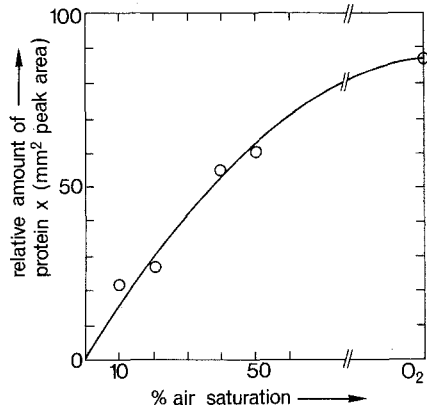
Fig. 5. The effect of O_2 concentration on the expression of protein X. Cells were grown at the indicated oxygen concentrations and the relative amount of protein X was assayed via densitometric analysis of SDS–PAA gels of 100 μg protein for each growth condition. The cells were sonicated prior to electrophoresis.

in glucose/nitrate and glycerol/fumarate membrane vesicles (van der Plas *et al.*, 1983). The precipitation lines 12 and 27 correspond to fumarate reductase, and line v to succinate dehydrogenase (van der Plas *et al.*, 1983). This implies that during growth with glucose/O_2, the latter enzyme is repressed to a level below the limit of detection of this CIE technique. Formate dehydrogenase "H" and hydrogenase are not detected in glucose/N_2 vesicles. Most probably this is due to a too high pH of the growth medium and the absence of molybdate and selenate (Ruiz-Herrera and Alvarez, 1972; Thomas *et al.*, 1976).

The cytochrome spectra of glucose/O_2 and glucose/N_2 membrane vesicles confirm previous findings (Gray *et al.*, 1966a). The precipitation line (No. 7) that contains cytochrome oxidase and heme-associated peroxidase activity is most likely cytochrome d, since the reaction with TMPD has been shown to be specific for this cytochrome (Krantz and Gennis, 1982).

Table II demonstrates that the enzyme activity is not a good indication of the amount of a particular enzyme present in the membranes. Of the four dehydrogenases, three are present in significantly higher amounts anaerobically; however, their activity is higher in the aerobically grown cells. The exact ratio depends on the assay used; the largest discrepancies are observed with the oxidase assay. However, this assay is only valid if the dehydrogenase is the rate-limiting step during electron transfer from the substrates to oxygen. Similar differences between amount and activity have been observed for 6-phosphogluconate dehydrogenase. Schnaitman (1970) also suggested the existence of a discrepancy between enzyme activity and enzyme quantity on the basis of measurements of enzyme activity and analysis of SDS–PAA gels. One can only speculate on the reason for the discrepancy: the enzymes from cells grown anaerobically may have an intrinsically lower specific activity or a lowered stability (e.g., a higher sensitivity toward oxygen).

Protein X has not been positively identified. However, its location (Table

III) and dependence on oxygen concentration (Fig. 5) makes it very likely that protein X is involved in oxygen metabolism. Owen and Kaback (1979) concluded that D-lactate dehydrogenase dislocates slightly during the preparation of membrane vesicles (from the difference in net orientation between NADH-dehydrogenase and D-lactate dehydrogenase). A parallel argument applies to protein X: this protein also tends to become dislocated during cell lysis. However, the magnitude of the standard deviation in these experiments precludes a firm conclusion. Despite many efforts, we have not been able to correlate protein X with two enzymes involved in oxygen metabolism: catalase and peroxidase (tested according to Konings and Boonstra, 1977, and Thomas, 1976); also analysis of literature data has not given us an indication about the identity of protein X. One possibility would be superoxide dismutase; however, several differences between this enzyme and protein X exist (Keele *et al.,* 1970), of which the molecular weight is the most important one.

Crossed immunoelectrophoresis has the major disadvantage that only those proteins that are immunogenic are detected. This phenomenon was not very important in this study since essentially all protein bands disappeared upon adsorption with antibodies (Fig. 4, gels c–g).

Conclusion

The results of this study confirm Schnaitman's (1970) conclusion that no large qualitative differences between the membrane proteins of glucose/O_2 and glucose/N_2 vesicles exist. Only two proteins are exclusively present in *one* type of vesicle: nitrate reductase in glucose/N_2 membrane vesicles and protein X in glucose/O_2 membrane vesicles.

A much larger amount of proteins show quantitative differences between the two types of vesicles. In most cases these proteins are present in higher amounts in cells grown anaerobically.

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