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Resolution of Distinct Selenium-Containing Formate Dehydrogenases from *Escherichia coli*

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Formate dehydrogenase, a component activity of two alternative electron transport pathways in anaerobic *Escherichia coli*, has been resolved as two distinguishable enzymes. One, which was induced with nitrate reductase as a component of the formate-nitrate reductase pathway, utilized phenazine methosulfate (PMS) in preference to benzyl viologen (BV) as an artificial electron acceptor and appeared to be exclusively membrane-bound. A second formate dehydrogenase, which was induced as a component of the formate hydrogenlyase pathway, appeared to exist both as a membrane-bound form and as a cytoplasmic enzyme; the cytoplasmic activity was resolved completely from the PMS-linked activity on a sucrose gradient. When *E. coli* was grown in the presence of ^{75}Se-selenite, a 110,000-dalton selenopeptide, previously shown to be a component of the PMS-linked enzyme, was induced and repressed with this activity. In contrast, an 80,000-dalton selenopeptide was induced and repressed with the BV-linked activity and exhibited a distribution similar to the BV-linked formate dehydrogenase in cell fractions and in sucrose gradients. The results indicate that the two formate dehydrogenases are distinguishable on the basis of their artificial electron acceptor specificity, their cellular localization, and the size of their respective selenoprotein components.

Formate is metabolized by two alternative enzyme systems in *Escherichia coli* growing under anaerobic conditions. The formate-nitrate system is induced by nitrate and converts formate to CO_2 with the reduction of nitrate to nitrite (9, 20, 22). The formate hydrogenlyase system, which converts formate to CO_2 and H_2, is repressed by nitrate but becomes the major pathway of formate metabolism in its absence (12, 15, 22). Formate dehydrogenase catalyzes the first step in each of these pathways but it has not been established whether the same or distinct formate dehydrogenase components are involved.

It has been established that the formate dehydrogenase activities present under these two sets of conditions have differing electron acceptor specificities. The formate dehydrogenase formed in the absence of nitrate transfers electrons to benzyl viologen (BV) preferentially (12, 15), whereas that formed in the presence of nitrate preferentially uses methylene blue or phenazine methosulfate (PMS) as an electron acceptor (5, 12, 17).

The cellular location of these two activities is less well established. With cells grown in the absence of nitrate, Peck and Gest (15) observed that the BV-linked activity remained largely in the supernatant after high-speed centrifugation of crude extracts whereas methylene blue-linked activity was found exclusively in the pellet. In similar distribution experiments Gray et al. (8) found both methylene blue and BV-linked formate dehydrogenase in both membrane and supernatant fractions, whereas Ruiz-Herrera et al. (17) found both BV- and PMS-linked activities to be located exclusively in membrane fractions. Although these discrepancies have not been resolved, it seems likely that they involve inherent difficulties in defining precisely supernatant and membrane fractions by differential centrifugation.

Characteristics of certain *chl* mutants which lacked formate dehydrogenase have suggested a genetic relationship between these two activities. Ruiz-Herrera and DeMoss (18) found that approximately half of the mutants which lacked PMS-linked formate dehydrogenase retained the BV-linked activity as well as the ability to produce gas from formate. The remaining mutants lacked both formate dehydrogenase activities, and both were recovered by apparent single-step reversions. These results suggested that the two enzymes share at least one common component. Using a similar approach with *Salmonella*, Chippaux et al. (3, 4) have concluded

that a single formate dehydrogenase participates in both the formate-nitrate reductase and formate hydrogenlyase systems in that organism.

Although the PMS-linked formate dehydrogenase associated with the formate-nitrate reductase system has been purified and characterized (5) and several genes have been identified which control its formation (2, 7, 14), the relationship of this enzyme to that involved in the formate hydrogenlyase system has not been established. The studies presented here were initiated to determine whether the two activities can be clearly distinguished and resolved as an approach to defining their structural relationship and the molecular mechanisms involved in specifying a single enzyme reaction for two alternative pathways.

MATERIALS AND METHODS

Growth. *E. coli* PK27 (*thi*) was grown in L broth (11) in the presence or absence of 1% KNO_3. Both types of growth media were supplemented with sodium selenite and sodium molybdate to a final concentration of 10 μM for maximum formate dehydrogenase activities (12, 16). For isotope studies ^{75}Se-selenious acid was added to the growth media to a final concentration of 1 μM. Anaerobic conditions were maintained by sparging the cultures with 95% nitrogen and 5% CO_2. Turbidities were monitored with a Klett-Summerson colorimeter fitted with a no. 54 green filter. Unless otherwise stated, cultures were grown to a final turbidity of 80 Klett units.

Preparation of cell fractions. All steps in the fractionation procedure were carried out under argon. Cultures were harvested and washed once in degassed 50 mM Tris-hydrochloride (pH 8.0)-10% ethylene glycol-1 mM dithiothreitol (DTT). Crude extracts were prepared by passing a 10% cell suspension through a cold French pressure cell at 10,000 lb/in^2 followed by treatment with 5 μg of DNase per ml for 20 min in an ice bath and then centrifugation at 12,000 × *g* for 15 min to remove unbroken cells. This cell extract was then separated into membrane and supernatant fractions by ultracentrifugation at 150,000 × *g* for 2 h at 2°C. The pelleted membranes were resuspended in buffer to the original volume.

Enzyme assay procedures. Formate dehydrogenase activities were monitored spectrophotometrically by following the reduction of artificial electron acceptors. The reduction of BV was followed at 600 nm (5). The reduction of phenazine methosulfate was followed by coupling the reduction to dichlorophenolindophenol (19). Methyl viologen (MV)-nitrate reductase activity was determined by the procedure described by Showe and DeMoss (20).

Sucrose density gradients. All sucrose solutions were prepared on a wt/vol basis in 50 mM Tris-hydrochloride (pH 8.0)-1 mM DTT. A 0.5-ml amount of a cell extract was layered on a 10.4-ml 5 to 20% linear sucrose gradient prepared over a 1-ml 70% sucrose shelf. Centrifugation was carried out at 200,000 × *g* for 90 min at 2°C. Fractions of 0.5 ml were collected under argon and assayed for BV-formate dehydrogenase, PMS-formate dehydrogenase, and MV-nitrate reductase activities. For the radioactive studies, aliquots of each fraction were mixed with 10 ml of aqueous counting scintillant and counted in a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Rockville, Md.).

Gel electrophoresis procedures. Discontinuous sodium dodecyl sulfate (SDS)-polyacrylamide slab gel electrophoresis was carried out as described by Keesey et al. (10) using 7 to 15% gradient gels. Protein samples to be electrophoresed were combined with 80 mM Tris-hydrochloride (pH 6.8), 100 mM DTT, 2% SDS, 10% glycerol, and 0.004% bromophenol blue and heated 5 min at 100°C. Gel staining was carried out by the procedure of Anderson et al. (1). The molecular weights of ^{75}Se-selenopeptides were estimated by comparison to radioactive molecular weight standards after autoradiography of gels.

Protein determinations. Protein was estimated by the procedure of Lowry et al. (13).

Source of chemicals. Materials for preparing culture media were purchased from Difco Laboratories, Detroit, Mich. Chemicals employed in this study were purchased from Sigma Chemical Co., St. Louis, Mo. (DTT, SDS, MV, acrylamide, bis-acrylamide, and Trizma-Base), J. T. Baker Chemical Co., Phillipsburg, N. J. (sodium selenite and sodium molybdate), Calbiochem, La Jolla, Calif. (PMS and 2,6-dichlorophenolindophenol), Fisher Scientific Co., Pittsburgh, Pa. (ethylene glycol and potassium nitrate), New England Nuclear Corp., Boston, Mass. (radioactive molecular weight standards and ^{75}Se-selenious acid), BDH Chemical Ltd., Poole, England (BV), and Amersham Corp., Arlington Heights, Ill. (aqueous counting scintillant).

RESULTS

As observed in earlier studies (12, 17) the electron acceptor specificity of the formate dehydrogenase produced by *E. coli* under anaerobic conditions was different in cells grown in the presence or absence of nitrate. In crude extracts from cells grown with nitrate, the specific activity of PMS-linked formate dehydrogenase was 1.2 μmol/min per mg of protein and that of BV-linked formate dehydrogenase was 0.13 μmol/min per mg of protein, for a ratio of 9:1. This ratio was reversed in crude extracts of cells grown in the absence of nitrate, where the specific activities were 0.26 μmol/min per mg of protein for PMS-linked activity and 2.51 μmol/min per mg of protein for the BV-linked activity. Some loss of activity occurred when cells were broken, but the ratios were about the same whether the enzymes were assayed in either whole cells or crude extracts. When the crude extracts were subjected to differential centrifugation, the two activities were not exclusively localized in supernatant or membrane fractions (Table 1). Although the results of this type of analysis varied from one experiment to another, the activities were distributed fairly equally be-

TABLE 1. *Distribution of formate dehydrogenase activities between membrane and supernatant fractions*

Growth medium	Fraction	% Total activity in fraction		
		PMS-linked[a]	BV-linked[a]	Nitrate reductase[b]
L broth plus nitrate	Supernatant	36	50	20
	Membranes	64	50	80
L broth	Supernatant	54	61	18
	Membranes	46	39	82

[a] PMS-linked and BV-linked formate dehydrogenase activities determined as described in the text. Total recovery of both formate dehydrogenase activities from crude extracts ranged from 68 to 100%. For specific activities in crude extracts, see text.

[b] MV-linked nitrate reductase activity determined as described in the text. Specific activities of nitrate reductase were 1.31 μmol/min per mg of protein in the crude extract of nitrate-grown cells and 0.11 μmol/min per mg of protein in L-broth-grown cells. Recovery in the fractions was ca. 100%.

tween the membrane and supernatant fractions. This distribution of activities is in contrast to nitrate reductase which, as shown previously (9, 20), was localized mainly in the membrane fractions in these analyses.

The addition of nitrate to either type of extract did not change the distribution or relative levels of the BV-linked or PMS-linked formate dehydrogenase activities, indicating that the effect of nitrate on the alternative expression of these two activities is at the level of enzyme synthesis, through induction and repression. This rules out the possibility that nitrate directly interacts with the BV-linked formate dehydrogenase to convert this enzyme to a form that reacts specifically with PMS.

Because previous studies on the distribution of formate dehydrogenase in cell fractions have been contradictory, we examined culture age as a possible factor which might contribute to these apparent discrepancies. When cultures were harvested at different phases of the growth cycle, BV-linked activity appeared in the supernatant fraction in a significantly greater proportion (75%) after the culture was in the stationary phase for 3 h. In contrast, the PMS-linked formate dehydrogenase remained about equally divided between the membrane and supernatant fractions and nitrate reductase remained almost exclusively bound to the membrane fraction.

To compare the distribution of the two activities between membrane and supernatant fractions in a more rigorous way, we analyzed the distribution of these activities on sucrose gradients. Crude extracts of cells grown with and without nitrate were centrifuged through a 5 to 20% sucrose gradient over a 70% sucrose shelf. Under the conditions utilized, the membrane fraction sedimented to the 70% sucrose shelf and the supernatant proteins tended to remain close to the top of the gradient. With the extract from nitrate grown cells (Fig. 1) BV-linked formate dehydrogenase was located both in the membrane fraction at the shelf and near the top of the gradient. This latter peak of apparent soluble activity was clearly separated from the PMS-linked activity, which was equally divided between the membrane fraction at the shelf and a more heterogeneous fraction in the middle of the gradient. (It should be noted in Fig. 1 that the activity scale for PMS-linked activity is 10-fold higher than that for BV-linked activity). Nitrate reductase was mainly associated with the membrane fraction at the shelf, but was also found heterogeneously distributed in the middle of the gradient more or less parallel with the PMS-linked formate dehydrogenase activity. These results suggested that a significant proportion of the BV-linked formate dehydrogenase activity is a supernatant enzyme which is distinct from the PMS-linked enzyme. Furthermore, the heterogeneous distribution of the PMS-linked formate dehydrogenase and nitrate reductase in the middle of the gradient suggested the apparent supernatant component of these activities is associated with slowly sedimenting membrane fragments.

Similar results were obtained with a crude extract of cells grown in the absence of nitrate (Fig. 2). (In this case the activity scale for PMS-linked formate dehydrogenase and nitrate reductase have been expanded 10-fold and for BV-linked formate dehydrogenase they have been reduced 10-fold). The major part of the BV-linked activity remained toward the top of the gradient with a profile distinctly different from that of the PMS-linked activity. Again each activity had an apparent membrane component and both nitrate reductase and PMS-linked formate dehydrogenase sedimented in part as broad parallel peaks in the middle of the gradient.

All attempts to purify the BV-linked supernatant activity for a direct structural comparison with the previously characterized (5) PMS-linked enzyme have failed because of the extreme lability of the BV-linked activity. Addition of various potential stabilizing agents or the utilization of anaerobic or reducing conditions were ineffective. Although the enzyme activity was fairly stable in high concentrations of ammonium sulfate and was stored in this form, most activity was lost during the course of commonly applied fractionation procedures. Therefore, alternative approaches were sought for studying the structural relationship between the

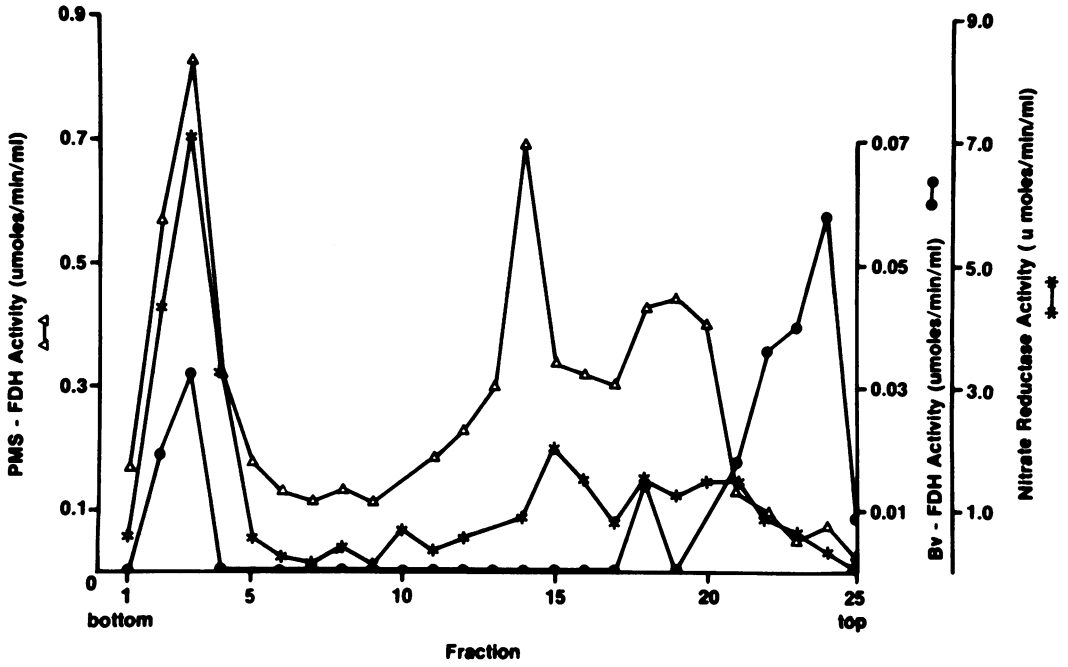

FIG. 1. *Distribution of enzyme activities from nitrate-grown cells on a sucrose gradient. A crude extract was prepared from nitrate-grown cells as described in the text, and a 0.5-ml portion of this extract was centrifuged through a 5 to 20% sucrose gradient over a 70% sucrose shelf at 200,000 × g for 1.5 h. Fractions were collected and assayed for enzyme activities as described in the text.*

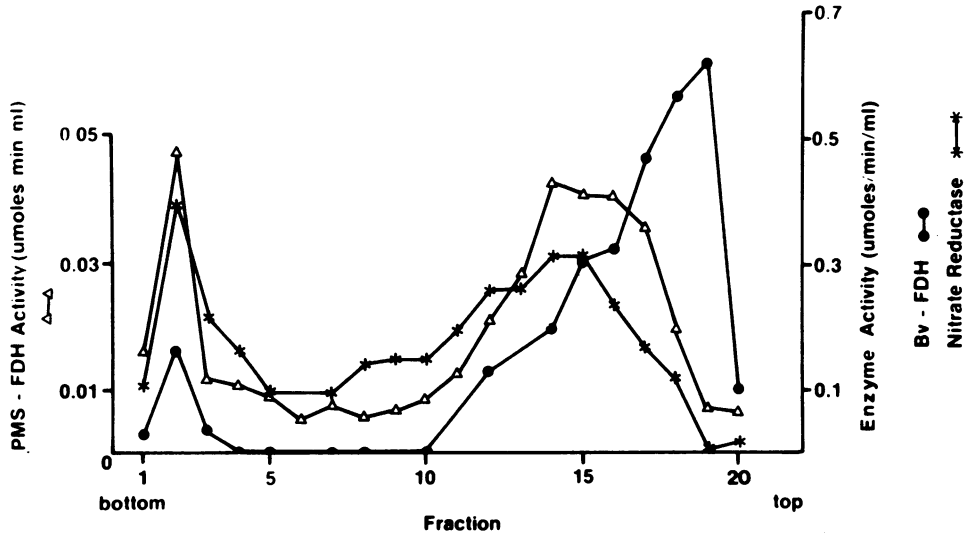

FIG. 2. *Distribution of enzyme activities from L-broth-grown cells on a sucrose gradient. With the exception that cells were grown in the absence of nitrate, the experiment was performed as described in the legend for Fig. 1.*

two formate dehydrogenases.

The formation of both formate dehydrogenases is dependent on an adequate supply of selenite in the growth medium of *E. coli* (12). Furthermore, Enoch and Lester (5) have shown that the PMS-linked enzyme is composed of three distinct subunits, one of which is a 110,000-dalton subunit containing covalently linked selenium. Therefore, another approach taken to study the relationship between the two enzymes

was to investigate whether the BV-linked formate dehydrogenase contained a similar or identical selenopeptide subunit. For this purpose *E. coli* was grown on broth containing ^{75}Se-selenite in the presence or absence of nitrate. The washed cell suspensions were treated directly with SDS and then subjected to electrophoresis on a polyacrylamide gel. Radioactive selenopeptides were identified after autoradiography, and their molecular weights were determined by comparison to radioactive molecular weight standards (Fig. 3). The SDS-treated extracts of nitrate-grown cells exhibited major radioactive bands of ca. 110,000 and 95,000 daltons with a very minor band of ca. 80,000 daltons. In contrast, the extract prepared from cells grown in the absence of nitrate exhibited an intense radioactive band corresponding to a selenopeptide of 80,000 daltons with only a minor component present at the 110,000-dalton position. Both types of extracts also contained a number of selenopeptides of less than 30,000 daltons. Thus, under conditions where the PMS-linked activity was repressed and the BV-linked activity increased, the 110,000-dalton selenopeptide disappeared and an 80,000-dalton selenopeptide accumulated.

Supernatant and membrane fractions were prepared from crude extracts of ^{75}Se-labeled cells, treated with SDS, and subjected to electrophoresis on a polyacrylamide gel. Autoradiography of the gel (Fig. 4) revealed that the 110,000- and 95,000-dalton selenopeptides in nitrate-grown cells were associated mainly with the membrane fraction while the major part of the 80,000-dalton selenopeptide in cells grown in the absence of nitrate remained in the supernatant fraction. The selenopeptides located in the region of the gel corresponding to less than 30,000 daltons remained in the supernatant in both types of extracts.

To examine further the relationship of the 80,000-dalton selenopeptide to the BV-linked formate dehydrogenase, a crude extract from cells grown in L broth plus ^{75}Se-selenite was centrifuged in a sucrose gradient (Fig. 5). The PMS- and BV-linked formate dehydrogenases were well separated with the BV-linked activity being close to the top of the gradient where most of the ^{75}Se was located. Electrophoresis of the individual fractions after treatment with SDS revealed that while the major part of the radioactivity was present in bands ranging in size from 10,000 to 30,000 daltons, the 80,000-dalton peptide was distributed in a profile similar to that of the BV-linked activity (Fig. 6). Some of the 80,000-dalton selenopeptide was also located at the 70% sucrose shelf. The small amount of the 110,000-dalton selenopeptide present was at the shelf and in the center of the gradient, corresponding to the PMS-linked activity, but not toward the top of the gradient where the BV-linked activity was located.

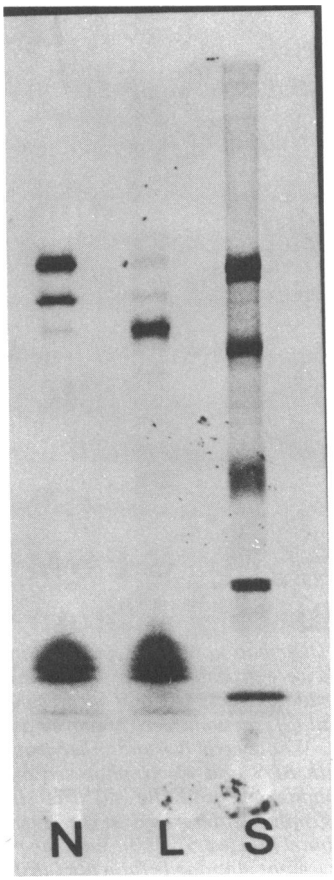

FIG. 3. *SDS-polyacrylamide gel patterns of selenoproteins exhibited by cells grown on ^{75}Se-selenite. Cells were grown in the presence (N) or absence (L) of nitrate in L broth. ^{75}Se-selenite (0.1 μM, 20 μC/μmol) was present in each medium. Washed cell suspensions were boiled in 2% SDS for 5 min before electrophoresis on SDS-polyacrylamide gels, and the molecular weights of ^{75}Se-selenoproteins were estimated after autoradiography as described in the text. The radioactive protein standards (S) used for estimating molecular weights of selenoproteins were phosphorylase a (97,000 daltons), bovine serum albumin (68,000 daltons), ovalbumin (43,000 daltons), carbonic anhydrase (29,000 daltons), and cytochrome c (12,500 daltons).*

DISCUSSION

The results presented here demonstrate that BV-linked and PMS-linked formate dehydrogenases are distinct and separable. The BV-linked enzyme was partly present in the supernatant in a form which sedimented as a homo-

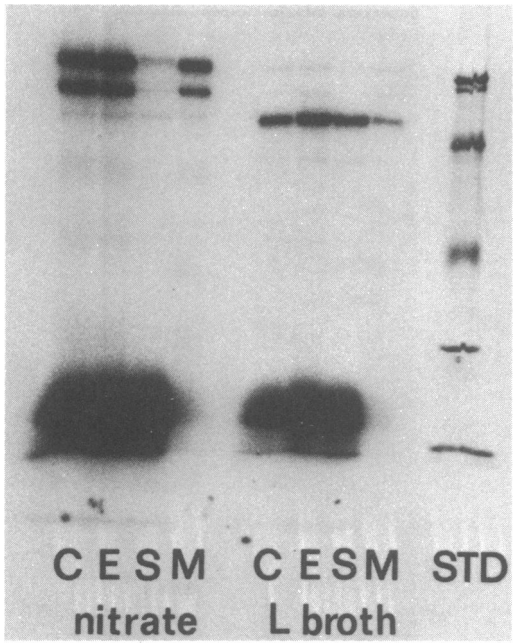

FIG. 4. *Distribution of selenoproteins in cell fractions. Cells were grown in the presence or absence of nitrate. Crude extracts (E) and membrane (M) and supernatant (S) fractions were prepared as described in the text. Whole cells (C) and other fractions were treated with SDS and electrophoresed on an SDS-polyacrylamide gel, and the gel was subjected to autoradiography as described in the legend for Fig. 3. The channel labeled STD contains the radioactive molecular weight standards indicated in Fig. 3.*

geneously distributed peak near the top of sucrose gradients. This form of the enzyme did not catalyze the PMS-linked reaction, as shown by the absence of this reaction in the fractions at the top of the gradient. The remainder of the BV-linked activity was membrane associated, based on the fact that it sedimented on the 70% sucrose shelf at the bottom of the sucrose gradient.

The PMS-linked formate dehydrogenase activity appeared to be exclusively membrane bound. Although only part of this activity sedimented with the membrane fraction with differential centrifugation, the apparent supernatant activity was distributed in a broad heterogeneous peak in the middle of the sucrose gradient. Nitrate reductase, a well-established membrane-bound enzyme (9, 20), was also found in small amounts distributed in a similar way on the sucrose gradients, indicating that membrane-derived fragments were present which fail to sediment by virtue of their size or composition. Tyhach et al. (21) have noted that phosphatidylserine decarboxylase is partly associated with a similar slowly sedimenting fraction in extracts of *E. coli* and concluded that this fraction represents membrane-like protein-lipid aggregates.

Although the two formate dehydrogenase activities are separable, the previous genetic studies with mutants defective in the formate-nitrate reductase pathway (18) or in the formate hydrogenlyase system (3, 4, 18) suggest that these two enzymes nevertheless share some component(s)

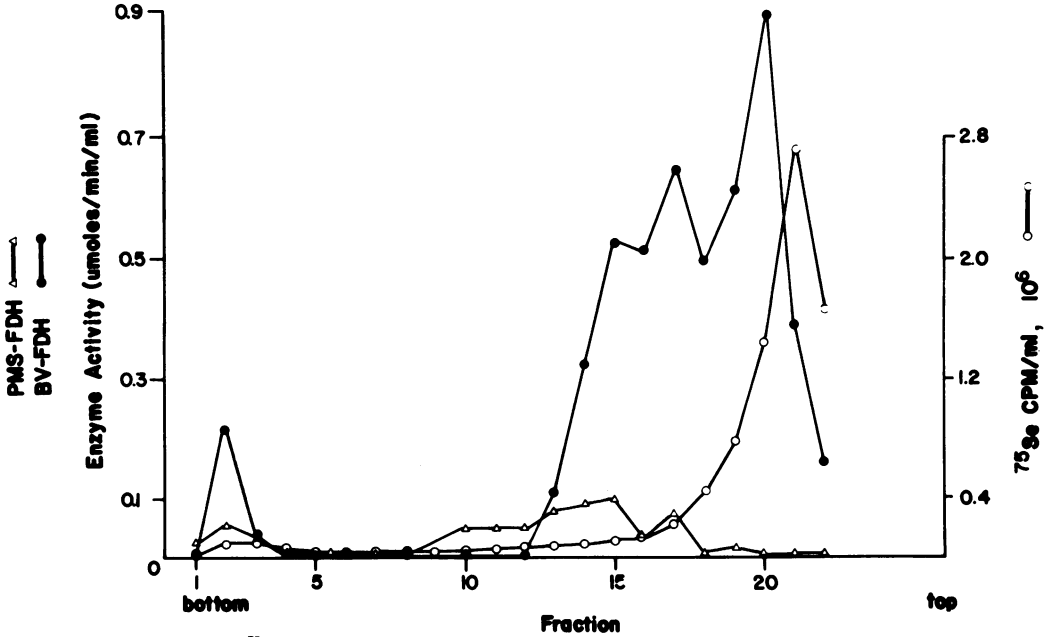

FIG. 5. *Distribution of ^{75}Se radioactivity on a sucrose gradient. With the exception that cells were grown on ^{75}Se-selenite, the experiment was performed as described in the legend for Fig. 2. An aliquot from each fraction was monitored for radioactivity as described in the text.*

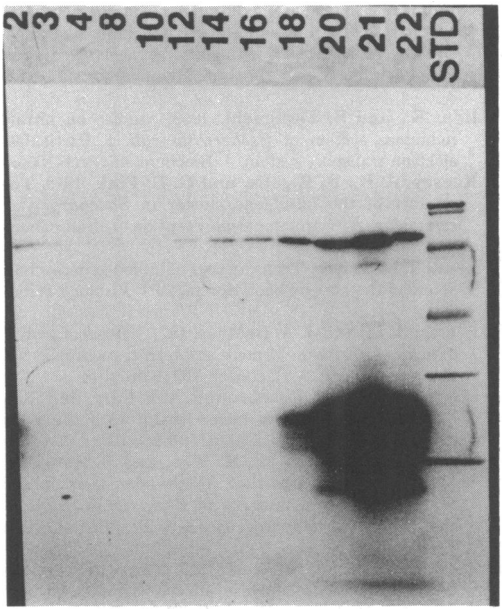

FIG. 6. *Distribution of ^{75}Se-selenoproteins on a sucrose gradient. Thirty-microliter portions of the sucrose gradient fractions from the experiment reported on in Fig. 5 were treated with SDS and electrophoresed on an SDS-polyacrylamide gel, and the gel was subjected to autoradiography as described in the legends for Fig. 3 and 4. The channels correspond to the sucrose gradient fraction indicated by the numbers at the top of the gel pattern. The channel labeled STD contains the radioactive molecular weight standards indicated in Fig. 3.*

in common. A logical candidate for this common component, based on the finding that selenium is required for the formation of both enzymes (12), was the 110,000-dalton selenoprotein subunit known to be a component of the PMS-linked formate dehydrogenase (5).

The results presented here demonstrate that the BV-linked formate dehydrogenase does not contain a 110,000-dalton selenopeptide identical to that present in the PMS-linked enzyme. This selenopeptide was present in reduced amounts when the PMS-linked enzyme was repressed and the BV-linked enzyme was induced, and insignificant amounts of this peptide were present in the sucrose gradient fractions which contained the BV-linked activity. Instead, an 80,000-dalton selenopeptide accumulated when the BV-linked formate dehydrogenase was induced, and it was distributed in the cell fractions in a similar way to the BV-linked enzyme. While more direct evidence is required to prove that the 80,000-dalton selenopeptide is a subunit of the BV-linked formate dehydrogenase, their similar behavior in fractionation procedures, their parallel induction and repression, and the known requirement for selenium for enzyme formation provide strong support for this suggestion.

The results presented here show that *E. coli* produces relatively few selenopeptides. In addition to the 110,000- and 80,000-dalton peptides, several other selenopeptides were observed: a 95,000-dalton peptide and a group of smaller peptides ranging from 10,000 to 30,000 daltons. The smaller peptides appeared to be formed under all conditions of growth and were invariably located in the supernatant fractions. The presence of the 95,000-dalton selenopeptide was much more variable. It was formed in variable amounts relative to the other peptides in repeated experiments and was completely absent in others. However, the fact that it was invariably repressed when the 110,000-dalton selenopeptide was repressed and was invariably localized in the same fraction suggests that it is a proteolytic product of the 110,000-dalton peptide.

Although the two formate dehydrogenases and their respective selenopeptides appear distinct, the data here do not rule out the possibility that the 80,000-dalton selenopeptide is also formed by proteolytic cleavage of the 110,000-dalton subunit. Such a conversion could conceivably affect both the electron acceptor specificity and cellular distribution of the enzyme. Alternatively, the two formate dehydrogenases may be composed of structurally independent selenopeptides with the point of common genetic control (18) involving the mechanism of selenium incorporation. Thus, mutations in the selenium incorporation pathway would inhibit the formation of both, as well as other, selenoproteins.

The BV-linked formate dehydrogenase which is induced in the absence of nitrate under anaerobic conditions has been shown to be a component of the formate hydrogenlyase pathway (8, 12, 15). The terminal enzyme in the pathway, hydrogenase, is membrane bound (6, 15), and interaction with formate dehydrogenase occurs through several as yet uncharacterized carriers (15). Formate dehydrogenase could conceivably participate in the hydrogenlyase pathway either in a soluble form which transfers electrons to soluble or membrane-bound carriers or in a membrane-bound form complexed with the intermediate carriers and hydrogenase. The results presented here indicate that BV-linked formate dehydrogenase is present as both supernatant and membrane-bound forms under the conditions used to prepare cell extracts. This is in direct contrast to the PMS-linked enzyme which remains tightly bound to the membrane fraction or membrane-like particles. The relationship of the membrane-bound BV-linked activity to the soluble BV-linked activity or to the PMS-linked activity has not been established.

However, the major part of the membrane-bound BV-linked activity is not readily released from the membranes by the same procedure which releases most of the PMS-linked activity, and the BV-linked activity that is released exhibits an electrophoretic mobility different from that of the PMS-linked activity (Cox and DeMoss, unpublished data). The basis for the association of the BV-linked formate dehydrogenase with the membrane and the form of the enzyme which participates in the formate hydrogenlyase pathway, as well as the precise structural relationship, if any, between the PMS-linked and BV-linked enzymes, can only be determined by direct genetic and biochemical approaches.

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