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# Isolation of the sulphur reductase and reconstitution of the sulphur respiration of *Wolinella succinogenes* *

**I. Schröder, A. Kröger, and J. M. Macy** **

Institut für Mikrobiologie der J. W. Goethe-Universität Frankfurt am Main, Theodor-Stern-Kai 7, Haus 75A, D-6000 Frankfurt, Federal Republic of Germany

**Abstract.** *Wolinella succinogenes* can grow at the expense of sulphur reduction by formate. The enzymes involved in the catalysis of this catabolic reaction have been investigated. From the results the following conclusions are drawn: 1. The enzyme isolated as a sulphide dehydrogenase from the cytoplasmic membrane of *W. succinogenes* is the functional sulphur reductase that operates in the electron transport from formate to sulphur. 2. The enzyme ($M_r$ 200,000) consists essentially of one type of subunit with the $M_r$ 85,000 and contains equal amounts of free iron and sulphide (120 μmol/g protein), but no heme. It represents the first functional sulphur reductase ever isolated. 3. The electron transport chain catalyzing sulphur reduction by formate consists merely of formate dehydrogenase and sulphur reductase. A lipophilic quinone which mediates the transfer of electrons between enzymes in other chains, is apparently not involved. This is the first known example of a phosphorylative electron transport chain that operates without a quinone. 4. The same formate dehydrogenase appears to operate in the electron transport both with sulphur and with fumarate as the terminal electron acceptor in *W. succinogenes*.

**Key words:** Sulphur reductase – Sulphur respiration – Electron transport – Reconstitution – *Wolinella succinogenes*

Since the discovery of *Desulfuromonas acetoxidans* (Pfennig and Biebl 1976) many other bacteria were isolated, the catabolism of which is chracterized by the reduction of elemental sulphur to sulphide (Widdel 1988). With some of these bacteria it was made clear that sulphur reduction is coupled to phosphorylation (Macy et al. 1986; Paulsen et al. 1986; Gebhardt et al. 1985; Widdel 1988). However, the enzymology and the mechanism of the reaction remained unrevealed.

Fauque et al. (1979) reported that the soluble cytochrome $c_3$ of sulfate reducing bacteria catalyzed the reduction of sulphur in the presence of $H_2$ and soluble hydrogenase. Using a membrane preparation of *Desulfovibrio gigas*, Fauque et al. (1980) found very small specific activities of ADP phosphorylation with phosphate which appeared to be coupled to sulphur reduction. Paulsen et al. (1986) reported that the membrane fraction of *D. acetoxidans* catalyzed the reduction of sulphur by succinate. The endergonic reaction was driven by the hydrolysis of ATP. As the process was abolished by protonophores or an inhibitor of the reversible ATP synthase, it was interpreted as an example of reversal of electron transport phosphorylation.

*Wolinella succinogenes* was reported to sustain growth by reaction (a)

$$\text{Formate} + S \rightarrow CO_2 + HS^- \qquad \text{(a)}$$

(Macy et al. 1986). This suggested that the reaction was catalyzed by a membraneous electron transport chain and was coupled to phosphorylation. In this communication the composition of the chain was investigated, as a prerequisite for understanding the coupling mechanism.

## Methods

### *Preparation procedures*

*Growth of Wollinella succinogenes.* The bacteria were grown as described earlier (Macy et al. 1986). However the medium (1.5 l in a 2.5 l fermenter) contained 15 mM (instead of 5 mM) sodium acetate, 70 mM (instead of 10 mM) sodium formate, and 0.5 g/l yeast extract instead of glutamate. The culture was gassed by passing a mixture (80% $N_2$/20% $CO_2$) over the culture at a rate of 0.3 l/min. Under these conditions the bacteria grew at a doubling time of about 4 h.

The bacteria were harvested by centrifugation at 15,000 × *g* (30 min at 0° C). For separation of the sulphur, the sediment was suspended in a buffer containing 0.25 M sucrose and 50 mM potassium phosphate, pH 7.4. The suspension was centrifuged for 5 min at 500 × *g*. The sediment (sulphur) was used for measuring the activity of formate oxidation by sulphur (see below). The supernatant was centrifuged as before and the bacteria were sedimented from the resulting supernatant by centrifugation at 30,000 × *g* (10 min), and stored in liquid $N_2$.

*Isolation of sulphur reductase and formate dehydrogenase.* All steps were performed at 0° C. The buffers used in the isola-

* Dedicated to Professor F. Schneider (Philipps-Universität Marburg) on the occasion of his 60th birthday

** *Present address:* Dept. Animal Science, University of California, Davis, CA 95616, USA

*Offprint requests to:* A. Kröger

*Abbreviations:* DMN, 2,3-Dimethyl-1,4-naphthoquinone; DTT, dithiothreitol; MK, menaquinone (vitamin $K_2$); PMSF, phenylmethane sulfonylfluoride; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-glycine; Tea, triethanolamine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethane sulfonate

tion procedure for formate dehydrogenase contained 2 mM $NaN_3$ and 1 mM DTT in addition, and were flushed with $N_2$.

The thawed bacteria (10–20 g protein/l) were stirred for 30 min in a buffer (50 mM Tris-chloride, 10 mM EDTA, 1 mM PMSF, pH 8) containing 1 g/l lysozyme. After the addition of 15 mM $MgCl_2$ and 1 mg/l DNAse, stirring was continued for 15 min and the membrane fraction was sedimented by centrifugation (20 min) at $30{,}000 \times g$. The sediment was suspended in a solution containing 25 mM imidazol-chloride and 10 g/l Triton X-100 (pH 7.6) to give a protein/detergent ratio of 1 g/g. After stirring for 30 min, the Triton extract was obtained as the supernatant resulting from centrifugation at $170{,}000 \times g$ (20 min).

The Triton extract (50 mg protein) was layered on a chromatofocusing column (PBE 94 from Pharmacia, 30 ml, 25 mm diameter) which was equilibrated with 450 ml of a solution containing 25 mM imidazol-chloride and 0.5 g/l Triton X-100 (pH 7.4). After 30 ml of the equilibration buffer had been passed through the column, the enzymes were eluted. The elution medium contained Polybuffer 74 which was diluted 8-fold with $H_2O$ and contained 0.5 g Triton X-100/l. The pH was adjusted to 5.0 using HCl. The fractions containing the enzymes at sufficiently high specific activity (see Fig. 2) were pooled, concentrated 10-fold by centrifugal ultrafiltration through a Centricon-30 filter (Amicon Corp.) and stored in liquid $N_2$.

*Preparation of liposomes.* Soy bean phospholipids (20 mg, Sigma P 5638) were sonicated for 10 min in 1 ml (50 mM Tricine, pH 8.1) at room temperature, 40 W and 50% duty cycle using the Branson sonifier equipped with the microtip.

For the preparation of liposomes containing vitamin $K_1$, 20 mg of the phospholipids (see above) and 0.4 µmol of the quinone were solved in a mixture of 2 parts (v/v) $CHCl_3$ and 1 part methanol. After evaporation of the solvent using a stream of $N_2$ at room temperature, the residue was sonicated as described above.

The liposomal suspension was mixed with the enzyme preparations, and the Triton X-100 was removed by shaking the mixture with Amberlite XAD2 (Serva, 20 g/g Triton) for 30 min at 0°C. After separation of the Amberlite, the mixture was frozen in liquid $N_2$ and thawed at room temperature. The preparation was either stored in liquid $N_2$ or freeze-thawed again, before its enzymic activities were measured.

### *Analytical procedures*

*Activity of formate oxidation by sulphur.* The sulphur used in this reaction (reaction a) was isolated from the cultures of *W. succinogenes* (see Growth of *W. succinogenes*). The sediment obtained by the centrifugation at $500 \times g$ was suspended in 50 mM Tricine (pH 8.1) and centrifuged again at $500 \times g$. The sediment was suspended in the same buffer and sonified for 10 min at 0°C, 40 W and 50% duty cycle using the Branson sonifier equipped with the microtip. The sulphur content of the suspension was determined (Fliermans and Brock 1973) as 0.1 g/ml.

The enzymic activity was measured in 10 ml serum flasks sealed with butylrubber stoppers. The flasks contained 50 mM Tricine, 5 mM formate (pH 8.1, 37°C) and the sulphur preparation (0.3 mol S/l) described above. Oxygen was removed by evacuation and flushing with $N_2$, and the reaction was started by the addition of the enzyme preparation. After certain time intervals, 0.1 ml-samples were removed and mixed with 20 µl trichloroacetic acid (1 M). The mixture was centrifuged and formate was assayed (Bergmeyer 1970) in the supernatant. The unit of activity (U) was equivalent to the consumption of 1 µmol formate per min.

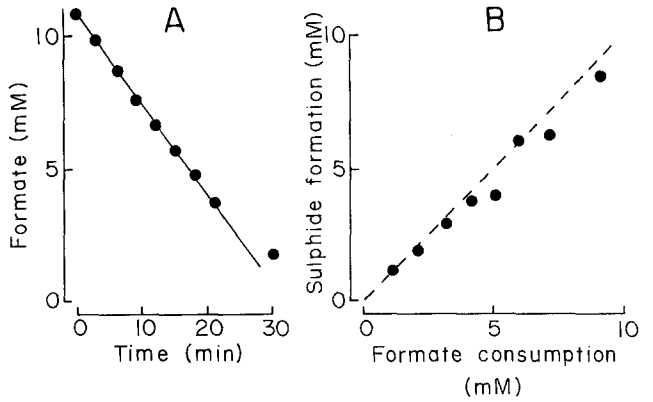


**Fig. 1 A, B.** Formate consumption and sulphide formation catalyzed by the membrane fraction of *Wolinella succinogenes* grown with sulphur as acceptor. The incubation medium contained 12.4 g/l membrane protein. The membrane fraction was prepared using the French press (see legend of Table 2). The *dashed line* (**B**) designates equimolarity of sulphide production and formate consumption

For the *determination of sulphide* (King and Morris 1967), the samples were mixed with zinc acetate 5.5% pH 4.7 instead of trichloroacetic acid.

*Sulphur reductase.* Sulphur reductase was assayed as the activity of DMN reduction by sulphide. The reaction mixture in a quartz cuvette ($d$ = 0.5 cm), with a butylrubber stopper contained 0.2 M Tea (pH 7.2) and 0.2 mM DMN at 37°C. Oxygen was removed by evacuation and flushing with $N_2$, and $Na_2S$ was added to give a final concentration of 0.1 M. This raised the pH to 7.9. The reaction was started by the addition of enzyme preparation, and the absorbance at 270–290 nm ($\Delta\varepsilon_{ox\text{-}red}$ = 15 $mM^{-1} \cdot cm^{-1}$) was recorded using a dual wavelength photometer. The unit of activity (U) corresponded to the reduction of 1 µmol DMN/min.

The *activities of formate oxidation by fumarate, DMN* (Unden and Kröger 1986) or *Methylene blue* (Kröger et al. 1979) were determined photometrically as described. The unit of activity was equivalent to the oxidation of 1 µmol formate/min at 37°C.

*Free iron* (Brumby and Massey 1967) *acid labile sulphur* (King and Morris 1967), *cytochrome c* (Francis and Becker 1984), *cytochrome b* (Kröger and Innerhofer 1976b) and *phospholipid* (Chen et al. 1956) were determined as described.

*Protein* was measured either using the biuret method (Bode et al. 1968) or with Amido black on nitrocellulose foil (Neuhoff et al. 1979).

## Results

### *Electron transport with sulphur as acceptor*

In the experiment of Fig. 1, the membrane fraction of *Wolinella succinogenes* grown on formate and sulphur was prepared using the French press. When this preparation was incubated with formate and sulphur, formate was consumed

**Table 1.** Comparison of enzymic activities in *Wolinella succinogenes* grown with sulphur or fumarate as acceptor. Enzymic activities of cell homogenates which were prepared by lysis of the bacteria. Lysis was brought about by stirring the bacteria (20 g protein/l) at 0°C in a buffer (pH 8) containing 50 mM Tris-chloride, 10 mM EDTA 1 mM $NaN_3$ and 0.1 g/l lysozyme. After 30 min 15 mM $MgCl_2$ and 4 mg/l DNAse were added and stirring was continued for 15 min

| Growth with | $H_2S \rightarrow$ DMN | Formate → DMN |
|---|---|---|
| | U/mg protein | |
| Sulphur | 16 | 8.1 |
| Fumarate | 0.50 | 1.6 |

and sulphide was produced. The average stoichiometric ratio was 0.92 mol sulphide/mol formate. The pH-optimum of the activity was at 8.1. The sulphur preparation was isolated from cultures of *W. succinogenes* and was used after sonication. Maximum activity of formate oxidation was observed with amounts which were equivalent to at least 0.2 mol sulphur/l. Sulphur prepared by acidification of polysulphide was less reactive.

The maximum specific activity of formate oxidation by sulphur observed (0.7 U/mg membrane protein) was about 5 times smaller than that calculated from the growth rate and the cell yield of the growing bacteria (see Table 6). This discrepancy was probably due to the limiting reactivity of the sulphur preparation used in the enzymic assay. The growing bacteria seemed to activate the sulphur prior to reduction.

*Induction of sulphur reductase*

The cell homogenate of *W. succinogenes* grown with sulphur catalyzed the oxidation of sulphide by DMN (Table 1). As demonstrated later on, this activity was due to the sulphur reductase. The specific activity of the homogenate was about 20 times higher than that of sulphur reduction by formate, in agreement with the view that the latter activity was limited by the reactivity of the sulphur preparation used (see above). Maximum activity was observed with Tea buffer at pH 7.9. The activity was about two times smaller, if Tris, Tricine, Hepes or glycylglycine were used as buffers. The $K_M$ for sulphide and DMN was measuredd as 21 mM and 60 μM respectively.

The specific activity of sulphide oxidation by DMN in sulphur-grown cells was about 30 times higher than that of the bacteria grown with fumarate (Table 1). This suggested that the enzyme was induced by the growth conditions. The specific activity of formate dehydrogenase was 5 times higher in cells grown with sulphur than with fumarate-grown bacteria.

*Localization of sulphur reductase*

In the experiment of Table 2, *W. succinogenes* grown with sulphur were homogenized using the French press. The resulting homogenate was centrifuged to give the membrane fraction and the soluble fraction. Most of the activity of sulphide oxidation was found in the membrane fraction, while 10% was present in the soluble fraction. The latter activity was not due to incomplete removal of membrane fragments, since formate dehydrogenase was absent from the soluble fraction. Thus *W. succinogenes* contains two sulphide dehydrogenases. The membraneous enzyme serves in the electron transport from formate to sulphur (see below). The function of the soluble sulphide dehydrogenase is not known.

*Isolation of sulphur reductase*

When the membrane fraction was treated with Triton X-100, more than 90% of the enzyme catalyzing sulphide oxidation by DMN was solubilized (Table 3). The specific activity of the Triton extract was 3.5 times higher than that of the membrane fraction. When the Triton extract was subjected to chromatofocusing, the enzyme eluted from the column in a single band with the peak at pH 6.4 (Fig. 2). In this step the sulphur reductase was separated from the formate dehydrogenase. Chromatofocusing caused a 3-fold increase in specific activity; the yield was 33%.

When the resulting preparation was subjected to the chromatofocusing step again, the specific activity decreased and more than half of the total activity was lost (not shown). This indicated that the enzyme was labile. Attempts to stabilize the activity with sulphide, dithiothreitol, EDTA, sucrose or NaCl were not successful. Purification by anion exchange chromatography, sucrose density gradient centrifugation or gel filtration also led to severe losses in enzymic activity.

*Properties of sulphur reductase*

Gel electrophoresis of the enzyme preparation in the presence of dodecylsulfate and subsequent staining with Coomassie blue suggested that more than 85% of the protein consisted of a polypeptide with the $M_r$ 85,000. In addition small amounts of peptides with $M_r$ 110,000, 60,000 and 54,000 were visible. The peptide with the $M_r$ 110,000 probably represented the bigger subunit of the formate dehydrogenase (Kröger et al. 1979). As judged from the stain and the specific activity of formate dehydrogenase, this peptide represented less than 10% of the total protein of the preparation. The second step of chromatofocusing caused the complete loss of the smaller $M_r$ peptides, while part of the enzymic activity was recovered. This suggested that the peptide were not essential for activity. The preparation did not contain appreciable amounts of fumarate reductase (Unden et al. 1980), hydrogenase (Unden et al. 1982), nitrite reductase (Schröder et al. 1985) or ATPase (Bokranz et al. 1985). These enzymes were earlier found in relatively large amounts in the membrane of *W. succinogenes* grown on formate and fumarate.

The $M_r$ of the protein of the active sulphur reductase was calculated as 200,000 from the sedimentation coefficient, the Stokes radius and the amounts of Triton X-100 (0.37 g/g protein) and phospholipid (0.03 g/g protein) which were bound to the protein (Tanford et al. 1974; Bokranz et al. 1985). The sedimentation coefficient (10.1 S) was determined using sucrose density gradient centrifugation (Martin and Ames 1961) and the Stokes radius (5.8 nm) was obtained by gel filtration (Bokranz et al. 1985). These properties suggested that the active enzyme consists of the dimer of the $M_r$ 85,000 subunit. The preparation contained 120 μmol/g protein of free iron and sulphide. This would be consistent with the presence of one or two iron-sulphur clusters per

**Table 2.** Cellular localization of sulphur reductase and formate dehydrogenase in *Wolinella succinogenes* grown with sulphur. Bacteria (20 g protein/l) were suspended in a buffer containing 0.25 M sucrose, 30 mM potassium phosphate, 2 mM EDTA (pH 7.4, 0°C) and passed 3 times through the French press at 0.1 MPa. The suspension was stirred for 15 min after the addition of 1 mg/l DNAse, 10 mM $MgCl_2$ and 1 mM PMSF and centrifuged for 5 min at 5,000 × *g*. The supernatant (cell homogenate) was centrifuged for 30 min at 170,000 × *g* to give the membrane fraction (sediment) and the soluble fraction (supernatant)

| Preparation | $H_2S \rightarrow$ DMN | | Formate → Methylene blue | |
|---|---|---|---|---|
| | U/mg protein | U | U/mg protein | U |
| Cell homogenate | 16 | 2685 | 25 | 4489 |
| Membrane fraction | 19 | 2050 | 46 | 4071 |
| Soluble fraction | 4.3 | 241 | < 0.1 | < 1 |

**Table 3.** Purification of the sulphur reductase of *Wolinella succinogenes* grown with sulphur. The purification procedure is described in the Methods section. The preparation obtained after chromatofocusing represents the fractions designated by the first bar in Fig. 2

| Purification step | $H_2S \rightarrow$ DMN | |
|---|---|---|
| | U/mg protein | U |
| Membrane fraction | 19 | 4248 |
| Triton extract | 67 | 3933 |
| Chromatofocusing | 186 | 851 |

**Table 4.** Purification of the formate dehydrogenase of *Wolinella succinogenes* grown with sulphur. The purification procedure is described in the Methods section. The preparation obtained after chromatofocusing represents the fractions designated by the second bar in Fig. 2

| Purification step | Formate → Methylene blue | |
|---|---|---|
| | U/mg protein | U |
| Membrane fraction | 17 | 3402 |
| Triton extract | 57 | 3302 |
| Chromatofocusing | 450 | 2121 |

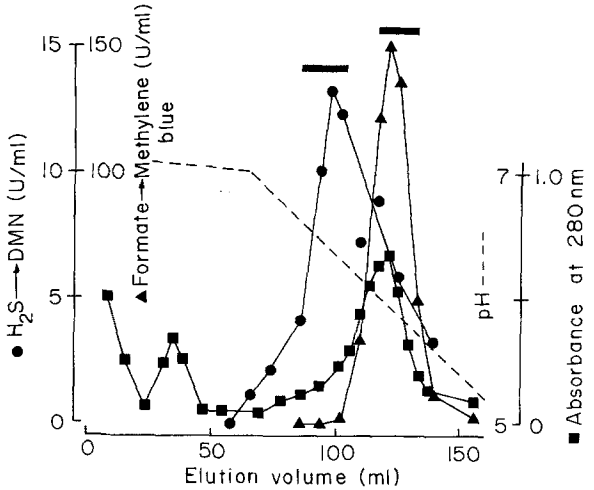


**Fig. 2.** Chromatofocusing of the Triton extract of the membrane fraction derived from *Wolinella succinogenes* grown with sulphur. The absorbance at 280 nm of the fractions was corrected for the content of Triton X-100. The corrected values were taken as a measure of protein content. The bars designate the fractions representing the isolated sulphur reductase (Table 3) and formate dehydrogenase (Table 4)

subunit. Cytochrome b and c as well as covalently bound FAD were absent from the preparation. From the increase in specific activity (10-fold) and the $M_r$ (200,000), the enzyme content of the membrane was estimated as 0.25 µmol/g membrane protein assuming that 50% of the enzyme had lost activity during isolation.

*Properties of formate dehydrogenase*

A formate dehydrogenase was earlier isolated from *W. succinogenes* grown with formate and fumarate (Kröger et al. 1979; Unden and Kröger 1986; Unden et al. 1983). This membraneous enzyme was found to consist of a peripheral part made up of the dimer of the $M_r$ 110,000 subunit which contained molybdenum, free iron and sulphide. The integral part consisted of cytochrome *b* ($M_r$ 25,000) with a midpoint of −224 mV. This cytochrome was essential for the electron transport from formate to MK (Unden and Kröger 1983). Some preparations of the enzyme contained a $M_r$ 20,000 polypeptide in addition.

The higher specific activity of formate dehydrogenase in cells grown with sulphur as acceptor (Table 1) could be explained in either of two ways. These bacteria might contain greater amounts of the formate dehydrogenase present in cells grown with fumarate. Alternatively a second formate dehydrogenase might be induced during growth with sulphur. To discriminate between these two possibilities, the formate dehydrogenase was isolated from cells grown with sulphur according to the procedure used for isolating the sulphur reductase (Table 4). More than 95% of the activity of methylen blue reduction by formate was solubilized by Triton X-100, while the specific activity was increased 3-fold. When the Triton extract was subjected to chromatofocusing, the formate dehydrogenase eluted as a single symmetrical band with the peak at pH 6.0. This band contained 90% of the total activity. The specific activity of the pooled main fractions of the band was 8 times higher than that of the Triton extract. Thus the membrane fraction of *W. succinogenes* grown with sulphur appeared to contain only one species of formate dehydrogenase.

After gel electrophoresis in the presence of dodecylsulfate and staining of the protein with Coomassie blue (not shown), 3 polypeptides ($M_r$ 110,000, 85,000 and 25,000) were detected in the preparation. The $M_r$ 85,000 peptide which amounted to less than 20% of the total protein, probably represented the subunit of sulphur reductase, since this

enzyme was not fully separated from formate dehydrogenase (Fig. 2). The $M_r$ of the residual polypeptides (110,000 and 25,000) were identical with those of the subunits of the formate dehydrogenase isolated from *W. succinogenes* grown with fumarate. Thus the formate dehydrogenase involved in the electron transport with sulphur as acceptor appeared to be identical with that operating in fumarate respiration. The enzyme content of the cytoplasmic membrane was estimated from the increase in specific activity (25-fold) to be 4% of the membraneous protein. Using the $M_r$ 250,000 (Kröger et al. 1979), the molar content was obtained as 0.16 μmol/g membrane protein.

*Incorporation of sulphur reductase into liposomes*

A suspension of sonic liposomes prepared from soy bean phospholipids was mixed with sulphur reductase (17 mg protein/g phospholipid). The mixture was agitated in the presence of Amberlite to remove the Triton X-100 which was associated with the enzyme preparation (Holloway 1973). Less than 10% of the enzyme and of the phospholipid was bound to the Amberlite under the conditions used (data not shown). After removal of the Amberlite, radioactive glucose was added and the mixture was freeze-thawed. Subsequent gel filtration revealed that more than 90% of the enzymic activity and of the phospholipid eluted at the void volume of the column together with a small proportion of the glucose (Fig. 3). This suggested that the enzyme was incorporated into liposomes. From the glucose/phospholipid ratio of the three main fractions, the average internal volume of the liposomes was calculated as 3.5 ml/g phospholipid. Assuming a spherical shape of the liposomes and considering the thickness of the membrane (6 nm), the diameter was calculated as 87 nm.

When gel filtration was done before freeze-thawing, part of the phospholipid and all the enzymic activity eluted beyond the void volume (not shown). The glucose/phospholipid ratio of the fractions containing most of the enzyme was 5 times smaller than that of the liposomes. This suggested that most of the enzyme was not integrated into liposomes unless the preparation was freeze-thawed, in agreement with earlier experience (Unden et al. 1983).

When a mixture of sonic liposomes, sulphur reductase and formate dehydrogenase was treated as described above, both enzymes were incorporated into liposomes (not shown). Using the liposomal size (87 nm diameter) and the enzyme/phospholipid ratio, it was calculated that the average liposome of the preparation used in the experiment of Table 5 contained 13 molecules of formate dehydrogenase and 5 molecules of sulphur reductase. When based on phospholipid, the liposomal contents of formate dehydrogenase (0.24 μmol/g) and sulphur reductase (0.10 μmol/g) were in the same order of magnitude as those of the bacterial membrane (see legend of Table 6 for comparison).

*Electron transport activity of the liposomes*

Liposomes containing both sulphur reductase and formate dehydrogenase catalyzed the electron transport from formate to sulphur (Table 5). In contrast, liposomes containing only one of the enzymes did not show electron transport activity. The activity of DMN reduction by formate was detected only in liposomes prepared with formate dehydrogenase; the activity of DMN reduction by sulphide was present only in liposomes containing sulphur reductase.

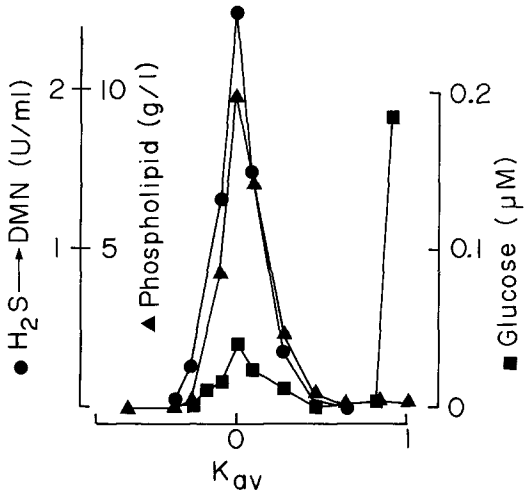


**Fig. 3.** Gelfiltration of liposomes prepared with sulphur reductase (17 mg protein/g phospholipid) and [$^{14}$C] glucose (1.07 μM and $2 \cdot 15 \cdot 10^{12}$ Bq/mol). The liposomal suspension (0.5 ml corresponding to 20 mg phospholipid) was layered on a Sepharose Cl-4B column (5 ml volume and 5 mm inner diameter). Equilibration and elution was done at 0°C using 50 mM Tricine buffer (pH 8.0) at a flow rate of 15 ml/h. The $K_{av}$ values were calculated from the elution volume ($V_e$), the total volume ($V_t$ corresponding to the elution volume at maximum glucose concentration) and the void volume ($V_o$ corresponding to elution volume at maximum phospholipid concentration) according to the equation $K_{av} = (V_e - V_o)/(V_t - V_o)$. Phospholipid was determined as phosphate after ashing (Chen et al. 1956)

To allow for a comparison with the enzymic activities of the bacterial membrane fraction, the liposomal activities were converted to turnover numbers. The turnover number of electron transport which was based on the content of sulphur reductase, was 2.5-fold smaller with the liposomes than with the bacterial membrane fraction. This difference was mainly due to the lower turnover numbers of the individual enzymes in the liposomes. Comparison of the turnover numbers indicated that sulphur reductase had retained approximately half and formate dehydrogenase one third of the original activity after isolation and incorporation into liposomes. The specific activity of electron transport was probably not decreased due to a significant proportion of liposomes that contained only one enzyme species. As judged from the estimated number of enzyme molecules present in an average liposome (see above), this proportion appeared to be negligible.

The activity of electron transport of liposomes containing vitamin $K_1$ (20 μmol/g phospholipid) was not higher than that of liposomes prepared without quinone. Vitamin $K_1$ substituted for MK in restoring the electron transport activity from formate to fumarate (Unden and Kröger 1982). MK was found to serve as an obligatory redox component in the chain catalyzing that reaction (Kröger and Innerhofer 1976a). From the phospholipid contents of the enzyme preparations and the MK/phospholipid ratio of the bacterial membrane, it was calculated that 50 nmol MK/g liposomal phospholipid was introduced into the liposomes together with the enzymes. The amount required for restoring a significant activity of the electron transport with fumarate was two orders of magnitude higher (Unden and Kröger 1982). It was considered unlikely that the activity of electron transport with sulphur observed with the liposomes would

**Table 5.** Enzymic activities of various liposomal preparations. Preparation (a) and (b) contained 0.1 g protein/g phospholipid. Preparation (c) and (d) contained 60 mg formate dehydrogenase protein and 20 mg sulphur reductase protein per gram phospholipid. Both enzymes were isolated from sulphur grown *Wolinella succinogenes*. The activities are given as turnover numbers which are based on the $M_r$ of sulphur reductase (20,000) or formate dehydrogenase (250,000). The turnover numbers of electron transport (formate → S) represent those of sulphur reductase. The contents of formate dehydrogenase (0.16 μmol/g protein) and sulphur reductase (0.25 μmol/g protein) in the membrane fraction (from sulphur-grown *W. succinogenes*) are given in the text

| Liposomes containing | Formate → S | $H_2S$ → DMN | Formate → DMN |
|---|---|---|---|
| | $10^3$ min$^{-1}$ | | |
| (a) Sulphur reductase | <0.01 | 1.3 | <0.02 |
| (b) Formate dehydrogenase | <0.01 | <0.03 | 12.4 |
| (c) Sulphur reductase + formate dehydrogenase | 1.0 | 4.0 | 9.5 |
| (d) Sulphur reductase + formate dehydrogenase + Vitamin $K_1$ | 1.1 | 5.2 | 9.1 |
| Membrane fraction | 2.6 | 9.2 | 27 |

**Table 6.** Comparison of the distance between the electron transport enzymes in the membrane and the distance migrated by MK or an enzyme during a turnover. The enzyme contents of the bacterial membrane refer to sum of formate dehydrogenase (0.03 μmol/g protein) and fumarate reductase (0.7 μmol/g protein) or of formate dehydrogenase (0.16 μmol/g protein) and sulphur reductase (0.25 μmol/g protein) in fumarate- or sulphur-grown bacteria, respectively. The distance between the enzyme molecules are calculated as the square root of the surface density of the enzymes assuming that only phospholipid (0.33 g/g protein) contributes to the membrane surface ($2.6 \cdot 10^6$cm$^2$/g phospholipid). The turnover numbers represent the ratio of the specific activities of formate oxidation by fumarate or sulphur and the sum of the enzyme contents. The specific activities are calculated from the doubling times (2 h with fumarate and 4 h with sulphur) and the cell yields (7 and 3.5 g cells/mol formate with fumarate and sulphur) on the basis that 1 g membrane protein corresponds to 4 g dry cells. The diffusion coefficients (Lenaz and Fato 1986) refer to MK (bacteria grown with fumarate) and to one of the enzymes (bacteria grown with sulphur). The distance migrated during a turnover of 2 electrons (d) is calculated as the square root of the ratio of the diffusion coefficient and the turnover number (Eq. 1)

| Bacteria grown with | Enzyme content (μmol/g phospholipid) | Distance between enzyme molecules (nm) | Turnover number ($s^{-1}$) | Diffusion coefficient (cm$^2$/s) | Distance migrated during a turnover (nm) |
|---|---|---|---|---|---|
| Fumarate | 2.1 | 14 | 74 | $10^{-6}$ | 1100 |
| Sulphur | 1.2 | 19 | 134 | $10^{-10}$ | 9 |

depend on such a small amount of MK. The following conclusions are drawn from the experiment of Table 5:

(i) The enzyme isolated as a sulphide dehydrogenase represents the sulphur reductase that operates in the electron transport from formate to sulphur (reaction a).

(ii) The electron transport chain is made up of formate dehydrogenase and sulphur reductase. MK is not involved.

The finding that MK is not involved in the electron transport with sulphur as acceptor, is in agreement with the fact that its redox potential ($E_o' = -75$ mV) is much more positive than that of the S/$H_2S$ couple ($E_o' = -245$ mV). To find out whether sulphur-grown *W. succinogenes* contain a more electronegative compound that may serve as a mediator between the enzymes, a methanol/petrol-ether extract of the membrane fraction was examined by thin-layer chromatography (not shown). The membrane of sulphur-grown cells was found to contain MK and Methyl-MK as that of the fumarate-grown bacteria (Collins and Fernandez 1984). Other lipophilic redox active compounds were not detected in the extract.

The apparent identity of the formate dehydrogenase of sulphur- and fumarate-grown bacteria was confirmed by measuring their activities in the electron transport both with fumarate or sulphur (not shown). This was done using liposomes that contained formate dehydrogenase from sulphur-grown cells together with vitamin $K_1$ and fumarate reductase (Unden et al. 1980) on one hand, and liposomes prepared with formate dehydrogenase from fumarate-grown bacteria together with sulphur reductase on the other. The turnover numbers of formate dehydrogenase in the two preparations were about the same.

## Discussion

Depending on the growth conditions, phosphorylation in *Wolinella succinogenes* is driven by the reduction of sulphur (reaction a) or fumarate (reaction b)

$$\text{formate} + \text{fumarate} + H^+ \rightarrow CO_2 + \text{succinate} \qquad \text{(b)}$$

with formate as the donor. The electron transport chain catalyzing reaction (b) is made up of formate dehydrogenase, fumarate reductase and MK (Kröger and Unden 1985). MK is reduced by the dehydrogenase and the quinol is reoxidized by the reductase (Kröger and Innerhofer 1976a). Each of the two enzymes appears to be randomly distributed in the bacterial membrane. As a consequence a dehydrogenase molecule is at the average 14 nm apart from a reductase molecule (Table 6). This distance is thought to be bridged by diffusion of the quinone and the quinol within the membrane. The distance ($d$) migrated by a quinone molecule

during a turnover can be estimated from its diffusion coefficient ($D$) according to the Einstein-Smoluchowsky equation [Eq. (1)],

$$d^2 = D \cdot t \quad (1)$$

when the time required for the turnover of 2 electrons is used as the migration time ($t$) (Kröger and Unden 1985; Lenaz and Fato 1986). This distance is found to be nearly 2 orders of magnitude greater than the average distance between the enzymes. Therefore, the electron transport activity with fumarate as acceptor is apparently not limited by the diffusion velocity of MK.

The electron transport chain catalyzing reaction (a) also consists of two enzyme species. However, a quinone is not involved. Therefore, the question arises as to how the reducing equivalents are transferred from formate dehydrogenase to sulphur reductase at the velocity of the overall electron transport. Two possibilities which cannot be discriminated by the results presented will be discussed:

1) The enzymes form a complex which is designed for short distance transfer of reducing equivalents.

This complex should be built similar to an electron transport enzyme which consists of different subunits and allows for rapid electron transport from one subunit to another [e.g. fumarate reductase of *W. succinogenes* (Unden and Kröger 1981)]. While these electron transport enzymes are not split into their subunits in the presence of Triton X-100, formate dehydrogenase can be separated from sulphur reductase under these conditions. This argues against the formation of a complex, but does not exclude it.

2) Formate dehydrogenase and sulphur reductase are randomly distributed in the membrane. The transfer of reducing equivalents from one enzyme to the other requires diffusion and collision of the enzyme molecules.

As shown in Table 6, the diffusion coefficient of the much bigger enzyme molecules is four orders of magnitude smaller as that of MK. As the distances between the enzymes as well as the turnover numbers of electron transport are similar with fumarate or sulphur as acceptor, the distance migrated by one of the enzymes during a turnover is two orders of magnitude smaller than calculated for MK. Actually the migration distance is close to the average distance between the enzyme within the membrane. This could mean that the electrón transport activity with sulphur is diffusion limited; each collision of the two enzymes would cause the transfer of two reducing equivalents. However, it is also possible that the collision frequency is even somewhat smaller than the turnover number. In this case more than two reducing equivalents should be transported after each collision of the enzyme molecules. This would mean that an electron transport complex is formed, the life time of which is given by the number of turnovers catalyzed.

Bacteria grown with sulphur contain 5 times more formate dehydrogenase than those grown with fumarate. As a consequence, the content of the dehydrogenase is close to that of the reductase, while fumarate reductase is in a 20-fold excess over formate dehydrogenase in the membrane of fumarate-gown cells. This difference in enzyme equipment may be a consequence of the different mechanism of electron transfer from the dehydrogenase to the reductase. The activity of fumarate reduction is not limited by the velocity of electron transfer from the dehydrogenase to the reductase, since the transfer is mediated by MK. In contrast sulphur reduction may be limited by the diffusion velocity of the enzyme molecules. In this case each enzyme molecule can reach its counterpart only once within a turnover. Therefore, the turnover number of each species of enzyme molecules is maximum with the same concentration of the other enzyme present.

In summary, the lack of a fast diffusing mediator in the electron transfer between the formate dehydrogenase and sulphur reductase requires complex formation of the two enzymes. The life-time of the complex could be as short as a single turnover or considerably longer. In the former case the activity of electron transport would be limited by the diffusion velocity of the enzyme molecules within the membrane.

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