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THE ORIENTATION OF THE SUBSTRATE SITES OF FORMATE DEHYDROGENASE AND FUMARATE REDUCTASE IN THE MEMBRANE OF *VIBRIO SUCCINOGENES*

ACHIM KRÖGER, ELISABETH DORRER and EDITH WINKLER

Institut für Physiologische Chemie, Physikalische Biochemie und Zellbiologie der Universität München, Goethestrasse 33, 8000 München 2 (F.R.G.)

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Summary

Formate dehydrogenase and fumarate reductase are involved in the electron transport phosphorylation system of *Vibrio succinogenes*. The orientation of the active sites of these enzymes in the cytoplasmic membrane of the bacterium was investigated with the aim of elucidating the mechanism of energy transduction. This was done by measuring the accessibilities of the enzymes to substrates, dyes and inhibitors both in cells and in cell-derived particles obtained with the French press.

1. After treatment of the cells with lysozyme and EDTA, followed by fractionation, both enzymes were found exclusively in the membranous fractions, while the periplasmic as well as the cytoplasmic fractions were devoid of both of the enzymic activities.

2. The sites of dye interaction of fumarate reductase were inaccessible to non-permeant dyes in cells, but were fully accessible in French-press particles. The K_m for succinate as measured with the permeant methylene blue as acceptor was increased ten fold on lysis of the cells. The K_m measured in the particles was similar to that of lysed cells and was not altered by lysis.

3. The rates of formate oxidation in the presence of non-permeant dyes and the K_m for formate were unaffected by cell lysis. On lysis of French-press particles, formate oxidation with both permeant and non-permeant acceptors was increased about three fold. The extent of stimulation was not altered by inhibition of the enzymic activities.

Abbreviations: benzyl viologen, 1,1′-dibenzyl-4,4′-bipyridylium dichloride; benzyl viologen sulfonate, *N,N′*-bis(4-sulfobenzyl)bipyridyl; diquat, 1,1′-ethylene-2,2′-bipyridylium dibromide; methyl viologen, 1,1′-dimethyl-4,4′-bipyridylium dichloride; morfamquat, 1,1′-bis(3,5-dimethylmorpholino-*N*-carbonylmethyl)-4,4′-bipyridylium dichloride; p-CMS, 4-chloromercuriphenyl sulfonate; NQNO, 2-(*N*-nonyl)-4-hydroxyquinoline-*N*-oxide; Hepes, *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonate.

4. Succinate oxidation by particles was fully inhibited by 4-chloromercuriphenyl sulfonate whereas that of cells was fully resistant. Formate dehydrogenase in cells was inhibited by 4-diazophenyl sulfonate when added together with formate. This compound also inhibited the enzyme in the particles when given in the absence of a lytic agent or after its addition. However, most of the enzyme remained active if the inhibitor was added before the lytic agent.

5. Fumarate and succinate were actively taken up by the cells from the medium, while formate did not even penetrate through the membrane of the bacteria.

6. It is concluded that the substrate and the dye-reactive sites of formate dehydrogenase face the outside, while those of fumarate reductase face the inside of the cytoplasmic membrane of cells of *V. succinogenes*. About 70% of the French-press particles were inverted with respect to the substrate and dye sites of the enzymes. The membrane is impermeable to formate and does not contain a specific transporter.

The electrogenic liberation of protons on the outside and the uptake of protons from the cytoplasm of the bacteria, which accompany electron transport, can be explained on the basis of the orientation of the substrate sites of the enzymes without net transport of protons across the membrane.

Introduction

Electron transport from formate to fumarate in *Vibrio succinogenes* is coupled to the phosphorylation of maximally 1 ADP/2 electrons [1—8]. Fumarate reduction by formate is associated with the generation of an electrochemical proton potential across the membrane [3—7] which possibly acts as the coupling device between electron transport and phosphorylation [9,10]. The proton/electron ratio is approximately 2 [3].

The apparent displacement of protons by electron transport in *V. succinogenes* has been previously explained by a mechanism which does not involve proton translocation across the membrane [5—7,11,12]. Similar mechanisms may also operate in other systems with proton/electron ratios not exceeding 2 [5,12—15]. The functioning of this mechanism in *V. succinogenes* requires an appropriate vectorial arrangement of the electron transport chain within the membrane (see Scheme II). An essential feature of this arrangement is the suitable orientation of the substrate sites of formate dehydrogenase and fumarate reductase, which were, therefore, investigated. For this purpose the reactions of the enzymes with various redox dyes were studied using different kinds of membrane preparations. This method could be applied successfully, since the enzymes have been characterized in a highly purified state [16,17] and since the sequence of the electron transport chain is well established [18,19].

Methods

Preparative procedures. Cultures of *V. succinogenes* [1], kindly provided by M.J. Wolin, were maintained and grown as described previously [1,16,18].

TABLE I

ASSAY CONDITIONS FOR THE VARIOUS ENZYMIC ACTIVITIES

The assays were performed as described in the text.

Activity	E'_0 of dye (mV)	Concentration (mM)	Wavelength (nm)	$\Delta\epsilon$ ($mM^{-1} \cdot cm^{-1}$)	Buffer	pH	Start with
Formate → diquat	−349 (20)	2	436	3.7	10 mM Tris	7.9	formate
Formate → morfamquat	−374 (20)	1	546	9.7	10 mM Tris	7.9	enzyme
Formate → phenazine methosulfate-2-sulfonate	80 (21) *	1	366	5.1	50 mM phosphate	7.0	enzyme
Reduced diquat → fumarate	−349	1	546	3.7	10 mM Tris	7.9	fumarate
Reduced morfamquat → fumarate	−374	0.5	546	9.7	50 mM phosphate	7.4	fumarate
Reduced methyl viologen → fumarate	−446 (20)	0.5	578	4.9	50 mM phosphate	7.0	fumarate
Reduced benzyl viologen sulfonate → fumarate	−350 (20) **	0.5	546	9.2	50 mM phosphate	7.0	fumarate
Anthrahydroquinone-2-sulfonate → fumarate	−225 (21)	1	334	5.1	50 mM Tris	7.9	fumarate
Succinate → methylene blue	11 (21)	0.2	578	17.1	10 mM Tris	7.9	enzyme

* Phenazine methosulfate.

** Benzyl viologen.

EDTA cells were prepared by incubating cells (about 2 g protein/l) in an anaerobic solution containing 0.5 M mannitol, 50 mM Tris and 10 mM EDTA at pH 8.0 and room temperature for 30 min. The EDTA cells were sedimented by centrifugation at 30 000 × *g* for 20 min, suspended in 0.5 M mannitol and 20 mM Hepes at pH 7.5, and kept at 0° C. The EDTA cells were lysed by freezing in liquid nitrogen and thawing with about 1 g Triton X-100 present/g of protein. Subsequently DNAase and $MgCl_2$ were added in order to decrease the viscosity of the suspension.

Spheroplasts were prepared by incubating the cells as described above. However, 0.1 g/l lysozyme was added after 10 min of incubation, and the suspension was stirred under nitrogen for 1 h. The spheroplasts were sedimented, suspended, and kept as described above for EDTA cells. The spheroplasts were lysed after centrifugation by suspending in an anaerobic solution containing 10 mM triethanolamine, 2 mM $MgCl_2$ and 4 mg/l DNAase at pH 7.5 and 0° C. The suspension was stirred under nitrogen for 1 h.

French-press particles were prepared by passing cells (5 g/l) in anaerobic solution containing 10 mM Tris, 1 mM NaN_3 and 4 mg/l DNAase at pH 7.9 and 0° C, through a French-press cell (capacity 35 ml; Aminco, Silver Spring, MD, U.S.A.) at 130 kg/cm^2 and at a flow rate of about 10 ml/min. The resulting suspension was diluted two fold with the same buffer and centrifuged at 12 500 × *g* for 30 min. The particles were separated from the supernatant by centrifugation at 50 000 × *g* for 30 min, suspended in 10 mM Tris and 1 mM NaN_3, pH 7.9, and kept at 0° C.

Melittin (purest available; Serva, Heidelberg, F.R.G.) and Triton X-100, in amounts of about 0.3 g/g protein and 0.01%, respectively, were added to the French-press particles directly in the test cuvette. Lysis of the particles (about 4 g/l) with phospholipase A_2 (2 g/l) was achieved by preincubation in an anaerobic solution containing 10 mM Tris, 1 mM NaN_3 and 2 mM $CaCl_2$ at pH 7.9 and 0° C for at least 30 min.

Enzymic activities. Activities with the dyes listed in Table I were measured in an Eppendorf photometer. Measurements were made at 25° C in a cuvette of 0.5 cm path length with the test solutions given in Table I. The solutions were bubbled with nitrogen for 2 min before the addition of the enzyme and then maintained under an atmosphere of nitrogen. The concentrations of formate and fumarate were 10 mM, while that of succinate was 20 mM. For measuring fumarate reduction with the viologen dyes, dithionite was added until the dyes were converted to the radicals. Anthrahydroquinone sulfonate was obtained from the quinone by reduction with KBH_4 in the test solution. The rates of ferricyanide reduction by formate and succinate as well as those of formate oxidation by fumarate [18], methylene blue and benzyl viologen [16] were measured as previously described. The unit of enzyme activity (U) represents the oxidation (reduction) of 1 μM formate or succinate (fumarate) per min.

Protein determination. Protein was measured using the biuret method with KCN [16].

Results

Localization of the enzymes

The study of the accessibility of enzymes to artificial reactants in various

TABLE II

LOCALIZATION OF THE ENZYMES

Spheroplasts were prepared and lysed as described in Methods. However, only 20 mg/l lysozyme were used. After lysis of the spheroplasts the suspension was centrifuged for 5 min at 3000 × *g* and the supernatant for 1 h at 100 000 × *g* (membrane fraction). The resulting supernatant (cytoplasmic fraction) and the supernatant obtained from the preparation of spheroplasts (periplasmic fraction) were concentrated about 20 fold by pressure dialysis. Menaquinone was determined as described previously [18].

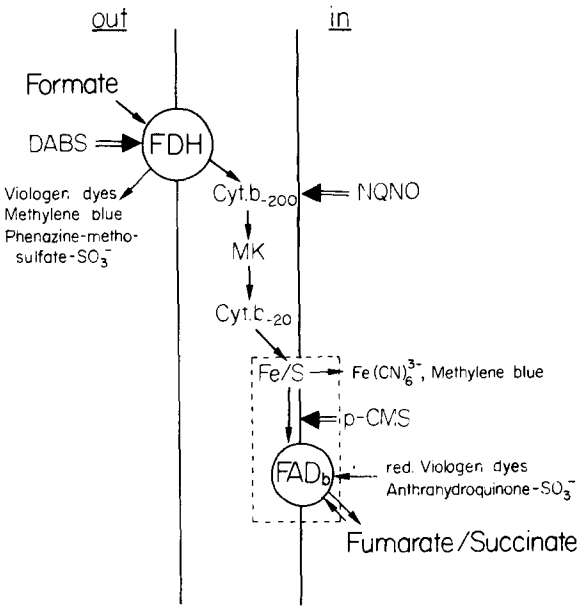
	Protein (%)	Formate → methylene blue activity		Succinate → methylene blue activity		Menaquinone content (μmol/g protein)
		Total (%)	Specific (U/mg protein)	Total (%)	Specific (U/mg protein)	
Cells	100	—	2.0	—	0.40	—
Periplasmic fraction	10	<1	—	<1	—	<0.5
Lysed spheroplasts	≡90	≡100	1.7	≡100	0.46	3.5
Sediment (3000 × *g*)	26	49	2.7	43	0.64	—
Membrane fraction	13	35	3.9	38	1.1	8.5
Cytoplasmic fraction	38	<1	—	<1	—	<0.5
Recovery	87	84	—	81	—	—

preparations of the bacterium initially requires unambiguous resolution of the possibility that more than one species of each enzyme exists in the different bacterial compartments (Table II). For this purpose the bacteria were treated with lysozyme in the presence of EDTA. The resulting spheroplasts were separated from the soluble proteins of the periplasmic space and lysed by osmotic shock. Unbroken cells were spun down at 3000 × *g* and the membrane fragments were separated from cytoplasmic proteins by centrifugation at 100 000 × *g*. Enzymic activities in the various fractions were measured with the permeable dyestuff methylene blue. This dye reacts directly with both enzymes [16,17] and enables the activity of enzyme molecules that may be enclosed by membranes to be recorded as well.

The periplasmic and cytoplasmic fractions were unable to reduce methylene blue in the presence of either formate or succinate, although these fractions contained 10 and 38%, respectively, of the bacterial protein. The activities of benzyl viologen reduction by formate and fumarate reduction by benzyl viologen radical were also not detected in these fractions (not shown). More than 80% of the enzymic activities of the spheroplasts were found in the membranous sediments. The specific activity with formate was 2.3 times and that with succinate 2.4 times greater in the membrane fraction than in the spheroplasts. As the menaquinone content of the preparation increased to the same extent (2.4 fold), it is concluded that both enzymes are present exclusively in the cytoplasmic membrane. The isolation procedures of the enzymes indicated that only one species each of formate dehydrogenase [16] and fumarate reductase [17] is bound to the membrane.

Accessibility of the enzymes in cells

The viologen dye diquat was found to react with the isolated enzymes



Scheme I. Interaction of formate, fumarate, succinate, redox dyes and inhibitors with the electron transport chain of *V. succinogenes*. FDH, formate dehydrogenase; encircled FAD_b designates the bigger subunit (*Mr* 79 000) of fumarate reductase which contains protein-bound FAD and iron-sulfur. The dotted box designates fumarate reductase.

formate dehydrogenase and fumarate reductase (Scheme I) and is known not to penetrate through the cytoplasmic membrane of bacteria [20]. The specific activities of diquat reduction by formate of cells, EDTA cells and in both intact and lysed spheroplasts of *V. succinogenes* were nearly identical (Table III). This indicates that the cell wall is permeable to diquat and that the formate dehydrogenase site which reacts with diquat is exposed to the outside of the cytoplasmic membrane of the bacterium.

The oxidation of reduced diquat by fumarate was equally slow with intact cells, EDTA cells and spheroplasts. Osmotic lysis of the spheroplasts increased

TABLE III

INFLUENCE OF CELL WALL DISINTEGRATION AND LYSIS OF THE CYTOPLASMIC MEMBRANE ON THE ACTIVITIES OF DIQUAT REDUCTION BY FORMATE AND OF DIQUAT RADICAL OXIDATION BY FUMARATE

Cells were lysed as described for EDTA cells in Methods. The enzymic activities were measured as described in Methods; 0.5 M mannitol was present in the test mixtures. Data are expressed as U/mg protein.

	Formate → diquat		Reduced diquat → fumarate	
	Intact	Lysed	Intact	Lysed
Cells	0.23	0.23	0.16	3.14
EDTA cells	0.28	0.27	0.17	7.4
Spheroplasts	0.29	0.26	0.16	8.1

TABLE IV

INFLUENCE OF LYSIS OF EDTA CELLS ON VARIOUS ELECTRON TRANSPORT ACTIVITIES

Donor	Acceptor	EDTA cells (U/mg protein)	
		Intact	Lysed
Formate	Benzyl viologen	0.99	0.93
Formate	Phenazine methosulfate sulfonate	0.55	0.58
Formate	Methylene blue	4.1	3.8
Formate	$Fe(CN)_6^{3-}$	0.44	2.1 *
Morfamquat radical	Fumarate	0.6	8.0
Methyl viologen radical	Fumarate	2.8	41
Benzyl viologen sulfonate radical	Fumarate	0.03	0.39
Anthrahydroquinone-2-sulfonate	Fumarate	0.12	2.8
Succinate	$Fe(CN)_6^{3-}$	0.11	2.1
Succinate	Methylene blue	0.45	0.43

* Lysed spheroplasts were used instead of lysed EDTA cells.

the specific activity of fumarate reduction 50 fold. This indicates that the site of fumarate reductase reacting with diquat faces the cytoplasm of the bacterium. Freezing and thawing of the EDTA cells in the presence of Triton released almost the same specific activity of fumarate reduction as lysis of the spheroplasts. The specific activity of cells frozen and thawed in the presence of Triton was only 39% of that of lysed spheroplasts. This was probably due to incomplete lysis of the cells. Triton had no effect on the activities with either formate or fumarate when added to the lysed spheroplasts (not shown).

The results obtained with diquat were confirmed with other redox dyes. Oxidized benzyl viologen [20], phenazine methosulfate sulfonate [22] and morfamquat [20] cannot penetrate the bacterial membrane and react directly with the isolated formate dehydrogenase (Scheme I). Lysis of the EDTA cells had no effect on the reduction of these dyes by formate (Tables IV and VII). Thus the site(s) of formate dehydrogenase reacting with these acceptors are oriented towards the outside of the cell. As expected, formate oxidation with the permeable methylene blue was also not stimulated by lysis of the EDTA cells (Table IV). The stimulation of formate-ferricyanide reduction on lysis is due to the fact that ferricyanide reacts preferentially with fumarate reductase, whereas the reaction with formate dehydrogenase is, by comparison, slow [16].

The radicals of morfamquat, methyl viologen [20] and benzyl viologen sulfonate, as well as anthrahydroquinone sulfonate, are impermeable dyes which react with isolated fumarate reductase (Scheme I). The increase (more than ten fold) of the activities of fumarate reduction with these donors on lysis of the EDTA cells (Table IV) demonstrates that the site of fumarate reductase reacting with these dyes is oriented towards the cytoplasm.

The site of interaction of ferricyanide with isolated fumarate reductase is different from that of the donors (Scheme I). As the 16-fold stimulation of succinate-ferricyanide reduction resulting from lysis of the EDTA cells shows, this site is also situated on the inside of the membrane of the cells. As expected,

no stimulation on lysis was found with the permeable methylene blue as acceptor of succinate oxidation.

Accessibility of the enzymes in French-press particles

By passage of *V. succinogenes* through a French press and subsequent differential centrifigation, Reddy and Peck [8] obtained a particle preparation which catalyzed the phosphorylation of external ADP as a function of fumarate reduction by hydrogen. The high yield of ATP indicated that a considerable part of the preparation consisted of closed vesicles, with the active site of ATPase exposed to the external medium, suggesting that the phosphorylating particles were inverted. This was confirmed by accessibility studies of formate dehydrogenase and fumarate reductase in these particles (Table V). The activity of fumarate reduction by the diquat radical was high with the intact particles, in contrast to the situation with cells (Table III) and was not stimulated either by the addition of Triton or melittin or by treatment with phospholipase. The same result was obtained after freezing and thawing of the particles in the presence of Triton (not shown). This indicates that the viologen site of fumarate reductase, which faces the inside of the cells, is accessible to the impermeable dye in these particles.

The oxidation of formate by diquat was stimulated three fold on treatment of the particles with Triton either with freezing and thawing (not shown) or without (Table V). The same extent of stimulation was brought about by melittin or by incubation of the particles with phospholipase. The addition of Triton after treatment with melittin or phospholipase did not increase the activity any further (not shown). This indicates that about 2/3 of the viologen sites of formate dehydrogenase in the particles are inaccessible to diquat. It is concluded that about 2/3 of the preparation consists of vesicles which are inverted with respect to the orientation of formate dehydrogenase and fumarate reductase. In the residual 1/3 of the preparation, both formate dehydrogenase and fumarate reductase appear to be accessible to impermeable dyes. In this respect these particles were similar to membrane fragments obtained by osmotic lysis of spheroplasts [18]. Melittin and phospholipase appear to make the membrane permeable to diquat in particles, but were much less efficient in this respect with cells, EDTA cells or spheroplasts (not shown).

The effect of melittin on other activities of the French-press particles is

TABLE V

EFFECT OF LYTIC AGENTS ON THE ACTIVITIES OF FORMATE OXIDATION BY DIQUAT AND OF FUMARATE REDUCTION BY DIQUAT RADICAL OF FRENCH-PRESS PARTICLES

The modes of application of Triton, melittin and phospholipase are described in Methods.

Lytic agent	Diquat radical → fumarate		Formate → diquat	
	Lysed (U/mg protein)	Lysed/ intact	Lysed (U/mg protein)	Lysed/ intact
Triton X-100	4.5	1.00	1.6	3.0
Melittin	4.5	1.03	1.7	3.1
Phospholipase A_2	4.3	0.97	1.5	2.8

TABLE VI

EFFECT OF MELITTIN ON VARIOUS ELECTRON TRANSPORT ACTIVITIES OF FRENCH-PRESS PARTICLES

The particles were lysed by the addition of melittin.

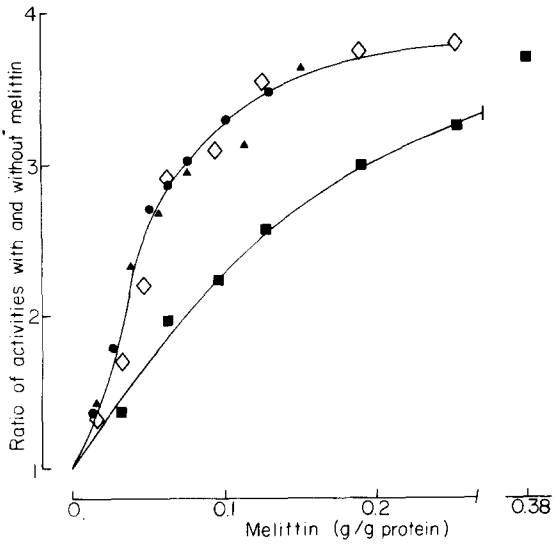
Reaction	Lysed (U/mg protein)	Lysed/intact
Reduced morfamquat → fumarate	6.7	1.0
Anthrahydroquinone sulfonate → fumarate	3.4	1.03
Succinate → $Fe(CN)_6^{3-}$	1.4	1.0
Formate → diquat	2.8	3.2
Formate → methylene blue	5.4	3.4
Formate → fumarate	3.7	3.4
Formate → $Fe(CN)_6^{3-}$	3.6	3.6

shown in Table VI. Results equivalent to those with melittin were obtained with Triton X-100 or phospholipase (not shown). The reduction of fumarate by the morfamquat radical or by anthrahydroquinone sulfonate and the oxidation of succinate by ferricyanide were not stimulated by the addition of melittin. Formate oxidation by methylene blue, ferricyanide or fumarate was stimulated by about the same extent as formate-diquat reduction. The stimulation of the former three activities can only be explained on the basis that melittin facilitates the permeation of formate and not of the acceptors through the membrane. The permeant methylene blue may either react with formate dehydrogenase directly, or accept electrons from formate via fumarate reductase. The site of fumarate reductase interacting with methylene blue is probably identical with that of ferricyanide reduction (Scheme I). This site is oriented towards the outside of the particles, as the response of succinate-ferricyanide reductase to melittin indicates. The substrate site of fumarate reductase was found to face the outside of the particles too (cf. Table VII). It is therefore concluded that the membrane is impermeable to formate and that the substrate site of formate dehydrogenase is oriented towards the inside of the inverted vesicles.

The activities of formate oxidation by fumarate, methylene blue and ferricyanide showed the same titration curves with melittin (Fig. 1). About 30 mg melittin/g protein were required for half-maximal stimulation of the three activities, while maximal stimulation was achieved with about 0.2 g. This suggests that the three reactions are limited by a common step. The titration curve of formate oxidation by diquat differed from that of the other three activities in that about double the amounts of melittin were required for half-maximal and maximal stimulation, respectively. This probably reflects the different requirements for permeation of the reactants. The inverted particles do not catalyze formate-diquat reduction unless the permeation of both reactants is facilitated by melittin. By contrast, the other three activities depend on the facilitated penetration of formate alone.

Accessibility of the enzymes to inhibitors

The isolated fumarate reductase preparation catalyzes the oxidation of



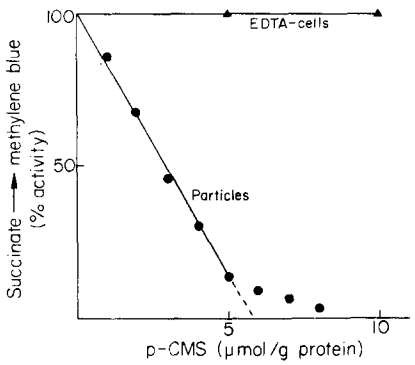


Fig. 1. Titration of various activities of formate oxidation of French-press particles with melittin. The increase in the activities was measured at each concentration of melittin. The activities without melittin in U/mg protein were: ■, 0.84 with diquat; ●, 1.0 with fumarate; ▲, 1.7 with methylene blue, and ◇, 0.90 with ferricyanide as acceptor.

Fig. 2. Titration of the succinate-methylene blue reductase activities of EDTA cells (▲) and French-press particles (●) with p-CMS. A specific activity of 100% corresponds to 0.30 and 0.76 U/mg protein with EDTA cells and particles, respectively.

succinate by methylene blue and this activity is effectively inhibited by p-CMS [17]. A similar result was obtained with particles, whereas the activity of cells was not inhibited (Fig. 2). As p-CMS is considered impermeant, the inhibition site of fumarate reductase appears to be oriented in the same way as the reaction sites of the dyes. The linearity of the titration curve up to 86% inhibition indicates that nearly all the inhibition sites of the particulate preparation are exposed to the outside.

The activity of formate-methylene blue reduction of EDTA cells was inhibited by relatively small concentrations of 4-diazophenyl sulfonate, provided that formate was present before 4-diazophenyl sulfonate was added (Fig. 3A). Thus more than 80% of formate dehydrogenase in the EDTA cells appeared to be accessible to the non-permeant inhibitor. Similar inhibition curves were obtained with the isolated formate dehydrogenase (not shown). The activity of French-press particles was similarly sensitive, both in the presence and in the absence of Triton (Fig. 3B). However, if Triton was added a few minutes after 4-diazophenyl sulfonate, a distinct stimulation was observed, and the difference between the activities after and before the addition of Triton was not affected by 4-diazophenyl sulfonate. This means that formate dehydrogenase in the inverted vesicles of the preparation is protected against inhibition unless lytic agents are present. The reaction of 4-diazophenyl sulfonate with 1-naphthylamine (not shown) revealed that the inhibitor was used up a few minutes after incubation with the particles in the presence of formate. The

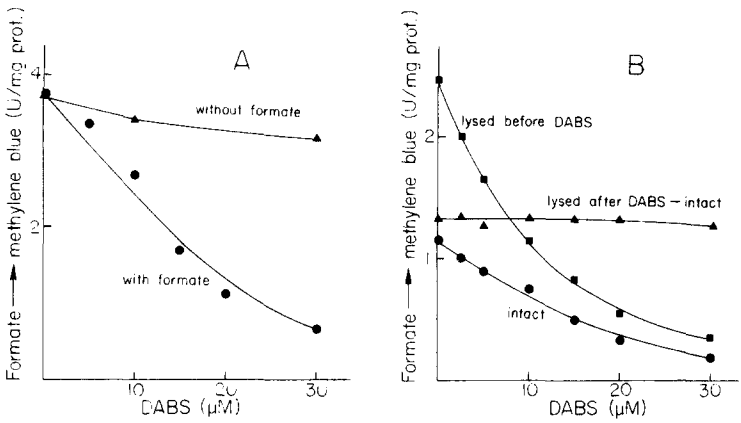


Fig. 3. Titration of the formate-methylene blue reductase activities of EDTA cells (A) and French-press particles (B) with 4-diazophenyl sulfonate (DABS). (A) EDTA cells (10 mg/l) were suspended in an anaerobic solution of 0.5 M mannitol and 10 mM Tris, pH 7.9, at 25°C, and the suspension was kept under N_2. ▲, DABS and methylene blue (0.2 mM) were added, and the reaction was started by the addition of 20 mM formate. ●, formate and DABS were added, and the reaction was started by the addition of methylene blue. (B) French-press particles (20 mg/l) were suspended in an anaerobic solution containing 10 mM Tris and 20 mM formate, pH 7.9, at 25°C, and the suspension was kept under N_2. ●, DABS was added and the reaction started by the addition of methylene blue; ■, as before, except that 0.01% Triton X-100 was present before the addition of DABS; ▲, as before, except that Triton was added after the reaction had been started by methylene blue.

inhibition of formate dehydrogenase in EDTA cells by radioactive 4-diazophenyl sulfonate did not give rise to specific labelling of the enzyme protein. The activities of fumarate reductase were also sensitive to 4-diazophenyl sulfonate in particles, but not in EDTA cells (not shown).

Effect of enzymic inhibition on the extent of the stimulation by lytic agents

The rate of formate penetration through the membrane of the particles was estimated by measuring the extent of stimulation of formate oxidation caused by melittin at various levels of inhibition (Fig. 4). The inhibition of formate dehydrogenase by azide is competitive with substrate [16]. The response of formate-methylene blue reduction of the particles in the presence and absence of melittin on progressive inhibition by azide is similar (Fig. 4A). Thus the extent of stimulation caused by melittin remains constant up to 90% inhibition. The same patterns were obtained by titration of formate-ferricyanide reduction with NQNO (Fig. 4B) and of formate-fumarate reduction by p-CMS (Fig. 4C). This shows that the extent of stimulation is independent of the acceptor of formate oxidation, of the inhibitor, and of the degree of inhibition. These results exclude the possibility that an appreciable part of formate oxidation activity in the untreated particles is due to a rate-limiting penetration of formate into the inverted vesicles. Rather, these results demonstrate that the penetration of formate is negligible by comparison with enzymic activity, and that the activities in the absence of lytic agents are due to membrane fragments, the formate dehydrogenase of which is exposed to the outside.

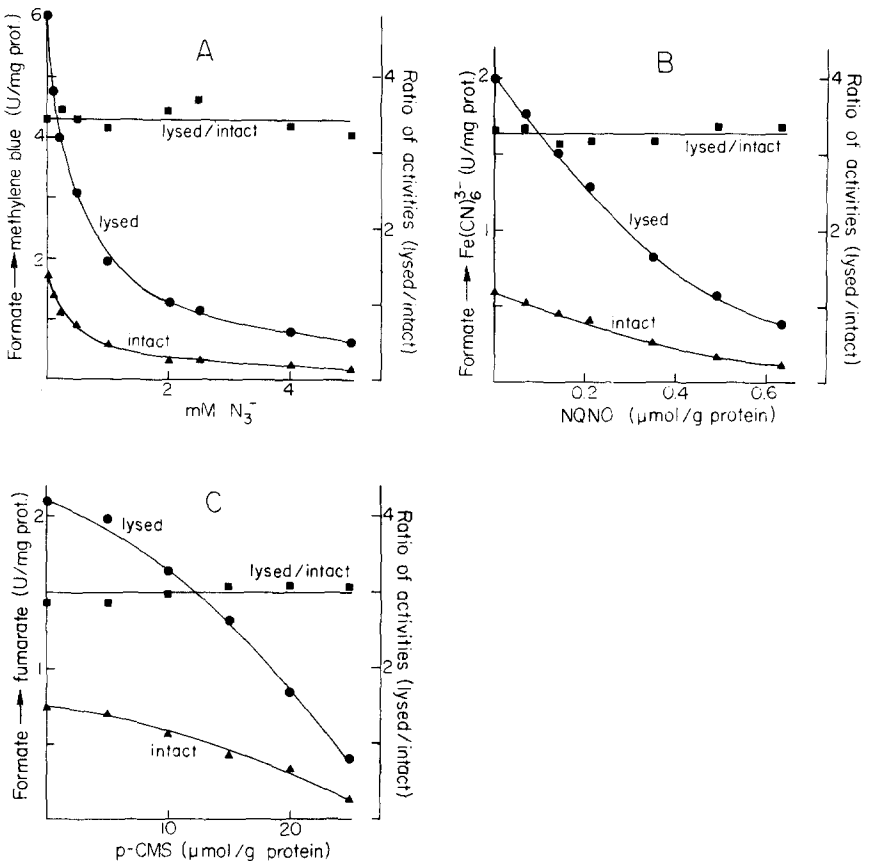


Fig. 4. Effect of inhibitors on the activities of formate oxidation by methylene blue (A), ferricyanide (B) and fumarate (C) with and without melittin. Activities were measured at each concentration of inhibitor before and after the addition of melittin.

Michaelis constants of the enzymes

It appears from the impermeability of the inverted vesicles to formate that the substrate site of formate dehydrogenase in *V. succinogenes* is oriented towards the outside of the cells. The substrate site of fumarate reductase should be exposed to the cytoplasm; otherwise the accessibility of the enzyme of inverted vesicles to fumarate and succinate would be difficult to understand. This view could be confirmed by measuring the K_m of the enzymes towards their substrates (Table VII). The K_m of fumarate reductase was measured for succinate with methylene blue as a permeant acceptor. This was necessary because the dye sites of fumarate reductase were found to be oriented to the insides of the cells, and a permeant donor was not available. Furthermore, the measurement of succinate oxidation is not disturbed by the activity of the fumarase of the cells, which would interfere with the fumarate reduction assay. It is likely that succinate interacts at the same site of fumarate reductase as fumarate itself.

The double-reciprocal plot of enzymic activity against succinate concentra-

TABLE VII

MICHAELIS CONSTANTS FOR SUCCINATE AND FORMATE IN METHYLENE BLUE REDUCTION OF EDTA CELLS AND FRENCH-PRESS PARTICLES

The activities were measured with 0.5 M mannitol in the test solution.

		EDTA cells		Particles	
		Intact	Lysed	Intact	Lysed
Succinate → methylene blue	K_m (mM)	0.3 (4.0)	4.2	4.4	4.4
	V_{max} (U/mg)	0.18 (0.45)	0.48	0.58	0.58
Formate → morfamquat	K_m (mM)	0.55			
	V_{max} (U/mg)	2.0			

tion gave straight lines with lysed cells and French-press particles, but was biphasic with unbroken EDTA cells (Table VII). This latter result suggests the presence of two sites of succinate interaction with EDTA cells. The K_m of the site of greater affinity for succinate (0.3 mM) was more than ten times smaller than that measured after lysis of the cells (4 mM). The V_{max} of the low-affinity site (0.45 U/mg protein) was 2.7 times greater than that of the high-affinity site (0.18 U/mg protein), and was nearly equal to the V_{max} measured with lysed cells. The K_m and V_{max} measured with French-press particles were similar to the values found in lysed cells, and were not affected by the addition of Triton. These results are interpreted to mean that the true K_m of fumarate reductase is measured either with French-press particles or after lysis of the cells. With whole EDTA cells at low substrate concentrations, the rate of succinate oxidation is probably limited by its transport through the membrane. The smaller K_m may, therefore, correspond to the transport of succinate. At higher concentrations of succinate the overall reaction may be limited by the activity of fumarate reductase.

The double-reciprocal plot obtained for formate oxidation by morfamquat was straight and was not affected by lysis of the EDTA cells. This result is in accordance with the view that the substrate site of formate dehydrogenase is exposed to the outside of the cell.

Permeation of formate and fumarate

When EDTA cells are separated by centrifugation from a suspension containing permeant or non-permeant solutes, they retain certain amounts of these solutes. Using radioactively labelled compounds, the amounts of formate and fumarate retained were measured and compared to the amounts of ^3H_2O and non-permeant solutes which were sedimented together with the EDTA cells. By this means it was attempted to decide whether formate and fumarate are capable of penetrating through the membrane or not (Table VIII). The experimental data are presented as the spaces (V) accessible to the solutes in the sediments at the concentrations of the external medium (first column) [23,24]. The spaces were obtained as the ratios of the contents of the solutes in the sediments and their concentrations in the corresponding supernatants. The space accessible to ferrocyanide (V_{FeCy}) is assumed to account for the amount of solute which adheres to the bacteria without penetrating the

TABLE VIII

UPTAKE OF FORMATE AND FUMARATE BY EDTA CELLS

EDTA cells (about 1 g/l) were suspended in an anaerobic solution containing 0.2 M mannitol, 0.15 M KCl, 20 mM Hepes, ^3H_2O and the compounds indicated at pH 7.6 and 5–6°C. After less than 1 min the suspension was centrifuged and the sediment separated from the supernatant. Both fractions were analysed for ^3H and ^{14}C. The spaces accessible to the solutes (V) and to water (V_{H_2O}) were calculated as the ratios of the contents in the sediments and the concentrations of the corresponding supernatants. The solute spaces were normalized to the average of the V_{H_2O}.

Additions	V (ml/g protein)	$(V - V_{FeCy})/(V_{H_2O} - V_{FeCy})$
^3H_2O	4.5	≡1.0
$Fe(^{14}CN)_6^{4-}$ (1 mM)	1.19	≡0
[^{14}C]Sucrose (1.7 mM)	1.93	0.22
[^{14}C]Fumarate (1 mM)	37.3	10.9
[^{14}C]Succinate (3 mM)	18.2	5.1
[^{14}C]Formate (1 mM)	1.14	−0.015
[^{14}C]Formate (1 mM) + fumarate (10 mM)	6.0	1.45
[^{14}C]Formate (1 mM) + fumarate (10 mM) + carbonic anhydrase (10 mg/l)	2.7	0.44

cytoplasmic membrane. V_{FeCy} is smaller than the space accessible to sucrose, although sucrose is generally regarded non-permeant. This may be explained by an absorption of sucrose by the EDTA cells on the outside of the cytoplasmic membrane. The values of V corrected for V_{FeCy} are considered to indicate the amounts of the solutes which have reached the cytoplasm of the EDTA cells. $V_{H_2O} - V_{FeCy}$ is assumed to represent the cytoplasmic space. The second column of Table VIII gives the concentrations of the solutes in the cytoplasm relative to that in the external medium.

The space accessible to fumarate was found to be 10.9 times greater than that of the cytoplasm. This means that fumarate is taken up and concentrated 10.9 times in the cytoplasm. Fumarate is probably taken up by exchange against the internal succinate which is formed by fumarate reduction. This is suggested by the observation that labelled succinate is also taken up and concentrated inside the EDTA cells (Table VIII), and that washed cells contain millimolar concentrations of succinate (not shown). The space accessible to formate is about equal to V_{FeCy}, indicating that the formate concentration in the cytoplasm is 0. The radioactivity of formate was concentrated 1.45 times in the EDTA cells when fumarate was added. However, most of the label was present as CO_2, since the cytoplasmic concentration of the radioactivity was only 44% of that of the medium in the presence of carbonic anhydrase. CO_2 is the product of formate oxidation by fumarate in *V. succinogenes* [5] and is permeant. It is likely that the label measured inside the EDTA cells with carbonic anhydrase present represents bicarbonate which was formed in the cytoplasm by hydrolysis of externally generated CO_2. The results indicate that fumarate is transported through the cytoplasmic membrane and formate is not. The membrane does not appear to be permeable to formate.

Discussion

Localization of the sites of dye reaction with the electron transport

The availability of preparations of isolated formate dehydrogenase [16] and

fumarate reductase [17] and detailed knowledge of the sequence of the electron transport chain of formate-fumarate reduction [18,19] permit the localization of the sites of action of the various dyes and inhibitors (Scheme I).

Oxidized viologen dyes (benzyl viologen, diquat and morfamquat) react with isolated formate dehydrogenase. Direct interaction with the membrane-bound enzyme is to be expected because of the low redox potentials of these dyes (Table I), which should not allow electron uptake from other electron transport components. This is confirmed by the insensitivity of formate-viologen reduction of membrane fragments to NQNO and p-CMS, which inhibit overall electron transport [16,18]. Formate-ferricyanide reduction of isolated formate dehydrogenase is relatively slow [16] while the activity of membrane fragments is both sensitive to NQNO (Fig. 4) and dependent on the presence of menaquinone [18]. It is therefore concluded that the reaction sites of ferricyanide are identical with formate and with succinate as donors. Methylene blue and phenazine methosulfate sulfonate can interact on the fumarate side of menaquinone on account of their positive redox potentials (Table I). However, their reactions with isolated formate dehydrogenase are fast [16], and the activities of formate oxidation with membrane fragments are insensitive to NQNO (not shown). This indicates that the preferential site of interaction of methylene blue and phenazine methosulfate sulfonate in formate oxidation is formate dehydrogenase itself.

Viologen radicals (of benzyl viologen, benzyl viologen sulfonate, methyl viologen, diquat and morfamquat) and anthrahydroquinone sulfonate react with isolated fumarate reductase, which contains only FAD and iron-sulfur [17,18]. Fumarate reduction by membrane fragments in the presence of these donors is insensitive to p-CMS and NQNO [18]. This indicates that the donors react preferentially with fumarate reductase, although their redox potentials (Table I) also allow interaction with more negative components of the electron transport chain. The relatively low activities of fumarate reduction by viologen radicals observed in cells, EDTA cells and spheroplasts (Tables III and IV) may be due to the reaction of the dyes with formate dehydrogenase.

Succinate oxidation by ferricyanide and methylene blue is also catalyzed by isolated fumarate reductase. The site of interaction of these dyes is different from that of the donors since succinate oxidation is strongly inhibited by p-CMS [17,18] (Fig. 2). The titres of inhibition of both activities of succinate oxidation of membrane fragments are equal (not shown) and identical with that of electron transport overall [18]. It is clear from the redox potential of the succinate/fumarate couple (30 mV) that the reaction site(s) of both acceptors is/are on the fumarate side of menaquinone. Therefore it is assumed that ferricyanide and methylene blue interact at the same site, probably an iron-sulfur centre. p-CMS probably attacks essential sulfhydryl groups of fumarate reductase [18].

From the experimental results presented and from the positions of the sites of interaction of the redox dyes and inhibitors with the electron transport chain as depicted in Scheme I, it is concluded that (1) the sites of formate dehydrogenase reacting with formate, dyes and 4-diazophenyl sulfonate are exposed to the outside, and (2) the sites of fumarate reductase reacting with fumarate, succinate, redox dyes and p-CMS face the inside of the cytoplasmic

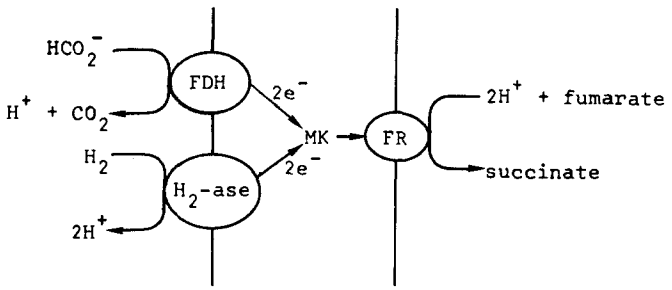
membrane of the cells. Fumarate and succinate are transported through the membrane, while a transporter for formate appears to be absent from *V. succinogenes*.

Relevance of enzyme orientation for phosphorylation

About 70% of the French-press particles are inverted with respect to the orientation of the reactive sites of the enzymes. The residual part of the preparation probably consists of non-vesicular membrane fragments. The particles are also inverted with respect to the orientation of the substrate site of ATPase [8]. Using French-press particles of *V. succinogenes*, Reddy and Peck [8] found that the reduction of fumarate by molecular hydrogen is coupled to the phosphorylation of external ADP. The maximal yield was 0.54 mol ATP/mol fumarate. The particles did not phosphorylate with formate instead of hydrogen as the donor for fumarate reduction, although the bacteria used were grown with formate as the growth substrate. However, phosphorylation of the endogenous ADP was observed with spheroplasts of *V. succinogenes* (up to 1 mol/mol fumarate) which was dependent on fumarate reduction by formate and sensitive to uncouplers [2—7]. This discrepancy can be explained by the different orientation of the substrate site of formate dehydrogenase spheroplasts and particles. Particles which are capable of energy transduction and phosphorylating external ADP are vesicular and inverted. These particles cannot oxidize formate because the substrate site of formate dehydrogenase faces the inside, and the membrane is impermeable to formate. On the other hand, particles oxidizing formate are incapable of energy transduction. Molecular hydrogen is expected to be oxidized by all kinds of particles, irrespective of the orientation of hydrogenase, since hydrogen can permeate through the membrane. This view is further confirmed by preliminary experiments in this laboratory, which indicate that hydrogen-fumarate reduction is associated with proton uptake by French-press vesicles, whereas formate-fumarate reduction is not. In contrast, fumarate reduction by hydrogen is coupled to proton liberation in the external medium with cells of *V. succinogenes* as is formate-fumarate reduction [3—7]. Formate-fumarate reduction is associated with an alkalinization of the cytoplasm [5—7].

Mechanism of generation of the electrochemical proton potential

The orientation of the substrate sites of formate dehydrogenase and fumarate reductase suggest that the electrochemical proton potential which is built up by formate-fumarate reduction [3—7] is generated according to the mechanism illustrated in Scheme II. The reaction of formate dehydrogenase with formate may release a proton and CO_2 into the external medium, and a further proton may be produced by hydrolysis of the CO_2. The transport of electrons from formate by the chain across the membrane to fumarate reductase would create a potential, and fumarate reduction on the inside would require the uptake of 2 protons/fumarate from the cytoplasm. Thus 2 protons/2 electrons would be liberated on the outside, and would disappear from the inside without movement of protons across the membrane. On the basis of the assumption that the substrate site of hydrogenase is oriented towards the out-



Scheme II. Mechanism of generation of the electrochemical proton potential proposed for fumarate reduction by formate and hydrogen in *V. succinogenes*. FDH, formate dehydrogenase; H_2-ase, hydrogenase; FR, fumarate reductase; MK, menaquinone.

side of *V. succinogenes*, an analogous mechanism may also operate in fumarate reduction by hydrogen.

The mechanism is consistent with the observed proton/electron ratio (1.6 [3]) and with the probable reaction mechanism of formate dehydrogenase and fumarate reductase, as well as with the sequence of the electron transport components. As formate dehydrogenase contains only electron acceptors (molybdenum and iron-sulfur [16]), it is likely that only the electrons derived from formate are accepted by the prosthetic group of the enzyme. It is unlikely that the protons required for fumarate reduction originate from the outside and are transported to fumarate along with the electrons, since the donor for fumarate reductase is a *b* cytochrome. Furthermore, the reaction sites of ferricyanide and p-CMS with fumarate reductase (probably iron-sulfur centres) are situated on the inside of cells (Table IV and Fig. 2).

If the mechanism given in Scheme II were operative in *V. succinogenes*, there would be no need for proton translocation by menaquinone [5,7]. As *b* cytochromes are probably the donor and acceptor for menaquinone (Scheme I) [19], proton exchange is intrinsically linked to its redox reactions. However, this does not necessarily imply vectorial net transport of protons across the membrane. The protons liberated by the oxidation of the hydroquinone in the steady state of electron transport may be simultaneously used up by the reduction reaction without ever leaving the membrane space. Alternatively, the protons may be exchanged only on one side of the membrane.

The redox potentials of both *b* cytochromes which are involved in electron transport (Scheme I) are pH independent between pH 6 and pH 8 (Kröger, A., unpublished results), in contrast to cytochrome *a* [25] and *b* [26,27] of mitochondria. Therefore proton translocation by cytochromes as envisaged by Wikström [28] and von Jagow et al. [29] can be excluded from the formate-fumarate reduction of *V. succinogenes*.

A mechanism of the type depicted in Scheme II was first proposed by Davies and Ogston [30] for mitochondrial respiration, long before it was shown that respiration is actually associated with the generation of an electrochemical proton potential [10]. The liberation of protons coupled to mitochondrial respiration in the presence of ascorbate and TMPD (tetramethylphenylenediamine) was later explained by a similar mechanism on the basis that ascorbate

is oxidized on the outside by cytochrome *c* and that oxygen reacts with cytochrome oxidase on the inside of the mitochondrial membrane [13]. The mechanism is probably also relevant to the respiration of lactate by yeast mitochondria and to the oxidation of sulfite by liver mitochondria, since the lactate dehydrogenases [31] and sulfite oxidase [32] are localized on the outside of the membrane, and the ATP yield is only one molecule/2 electrons. As the glycerol-1-phosphate dehydrogenase of liver and insect flight muscle mitochondria [24] and the external NADH dehydrogenase of yeast mitochondria [31] are oriented towards the outside, it is likely that similar mechanisms contribute to the generation of the proton potential. However, since the proton/2 electron ratios are greater than 2 in these cases, it is clear that proton translocation across the membrane is involved in addition. Garland and Jones [15] proposed that the proton potential associated with electron transport between ubiquinone and nitrate in *Escherichia coli* is created without proton translocation, although the orientations of the sites of proton liberation and disappearance have not yet been established [33]. Furthermore, the reduction of sulfate and CO_2 by hydrogen in sulfate-reducing and methanogenic bacteria is probably associated with the generation of a proton potential which may be built up according to a mechanism which is similar to that of Scheme I [14]. Finally, Hauska [34] proposed that an analogous mechanism contributes to the proton potential created by the electron transport associated with photophosphorylation.

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