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The electron transfer from hydrogenase and formate dehydrogenase to polysulfide reductase in the membrane of *Wolinella succinogenes*

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Abstract

Freeze-thawing of the membrane fraction of *Wolinella succinogenes* with sonic liposomes containing vitamin K-1 gave an apparently homogeneous particle preparation. The specific electron transport activity based on membrane protein of these particles with polysulfide as acceptor and formate or H_2 as donor decreased hyperbolically with the amount of liposomes fused to the membrane fraction, while the activities of formate dehydrogenase, of hydrogenase and of polysulfide reductase were not affected. The electron transport activity with H_2 did not decrease upon fusion to the membrane fraction of liposomes containing the isolated hydrogenase. Electron transport with fumarate as acceptor was nearly unaffected by the dilution of the membrane fraction with liposomes containing vitamin K-1. The results confirm that menaquinone is not involved in the electron transport from H_2 or formate to polysulfide, and argue against the existence of a stable electron transport complex within the membrane which is made up of a dehydrogenase and a polysulfide reductase molecule. Instead, the electron transport complex appears to be formed reversibly by diffusion and collision of the enzyme molecules.

Keywords: Polysulfide reductase; Electron transport; Formate dehydrogenase; Hydrogenase; (*W. succinogenes*)

1. Introduction

Wolinella succinogenes can grow at the expense of polysulfide reduction with either H_2 or formate as donor [1–3]. Both reactions (a) and (b) are coupled to

$$H_2 + S_n^{2-} \rightarrow H^+ + S_{n-1}^{2-} + HS^- \quad (a)$$

$$HCO_2^- + S_n^{2-} \rightarrow CO_2 + S_{n-1}^{2-} + HS^- \quad (b)$$

phosphorylation. The electron transport chains catalyzing reactions (a) or (b) appear to be made up of polysulfide reductase and either hydrogenase or formate dehydrogenase. The enzymes have been isolated and the corresponding genes sequenced [2,4–7]. Incorporation into liposomes of each dehydrogenase together with polysulfide reductase yielded proteoliposomes catalyzing reaction (a) or (b) in the absence of menaquinone, and the activities were not stimulated by the addition of vitamin K-1 [2,4]. Thus the electron transport from the dehydrogenases to polysulfide reductase was apparently not mediated by a respiratory quinone, in contrast to the majority of other systems of aerobic or anaerobic respiration, including the fumarate respiration of *W. succinogenes* [8].

The respiratory quinones are thought to transfer electrons by diffusion from the enzymes reducing quinone to those oxidizing quinol. The enzymes are thought to be randomly distributed in the membrane [9,10]. In the absence of quinones, two mechanisms are feasible for explaining the electron transfer between the enzymes. Stable electron transport complexes consisting of a dehydrogenase and a polysulfide reductase molecule may exist within the membrane which assure rapid electron transfer between the enzymes, and are cleaved by the detergent treatment applied in enzyme isolation. Alternatively, formation of the electron transport complexes may require diffusion and collision of the enzymes within the membrane. Experimental discrimination between the two mechanisms can be achieved by the incorporation of additional phospholipid into the bacterial membrane. This dilution of the enzymes should not affect the activity of electron transport, if a stable electron transport complex existed. In contrast, the activity should decrease with the phospholipid content, if the electron transport complex was reversibly formed from the enzymes.

Abbreviations: DMN, 2,3-dimethyl-1,4-naphthoquinone; $DMNH_2$, reduced DMN.

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2. Methods

2.1. Bacterial growth

W. succinogenes was grown with formate (80 mM) and fumarate (0.1 M) as described [2,11]. For growth with polysulfide, the bacteria were precultured on formate (80 mM) and 25 mM fumarate. After consumption of the fumarate, 7 mM sulfide was added by which the pH was shifted from 7.8 to 8.2, and solutions of $Na_2S_4O_6$ and of NaOH were continuously added for 5 h to a final concentration of 40 and 80 mM, respectively. Under these conditions growth was sustained by reaction (b) [12].

2.2. Cell fractionation

Bacteria harvested in the late exponential growth phase were suspended in anaerobic 'Tris buffer' (pH 8.0, 0°C) which contained 50 mM Tris-chloride and 1 mM each of dithiothreitol, malonate and NaN_3. Malonate and azide are competitive inhibitors of fumarate reductase and formate dehydrogenase, respectively, and stabilize the enzymic activities [13]. The suspension was passed through the French press at 100 bar and then centrifuged for 10 min at $10\,000 \times g$ to remove intact cells. The supernatant (cell homogenate), was centrifuged for 35 min at $150\,000 \times g$ to give the soluble fraction (supernatant) and the membrane fraction (sediment) which was suspended in the anaerobic 'Tris buffer' (5–10 g protein/l).

2.3. Fusion particles

Sonic liposomes containing vitamin K-1 (20 μmol/g phospholipid) were prepared from soy bean phospholipid (Sigma No. P5638) as described [14]. The final suspension in 'Tris buffer' contained 60 g phospholipid per liter and was stored in liquid N_2. The suspension of the membrane fraction (1 ml) was mixed with the appropriate amount of liposomes, and the mixture was frozen in liquid N_2 and subsequently thawed at 22°C. After about 20 min, freeze-thawing was repeated three times.

The liposomes used in the experiment of Fig. 2B were prepared by freeze-thawing of a mixture of sonic liposomes (40 mg phospholipid) containing vitamin K-1 (see above) and hydrogenase isolated from *W. succinogenes* (2.2 mg protein) [2].

2.4. Enzyme activities

Polysulfide reduction by formate or H_2 was recorded at 360 nm as described [2,12]. Fumarate reduction by formate or H_2 was recorded at 270–290 nm using a dual wavelength spectrophotometer as described [13]. The buffer (pH 7.4, 37°C) containing 50 mM potassium phosphate and 2 mM fumarate was alternately evacuated and flushed with H_2 or N_2 (with formate as substrate). The reaction was started by enzyme addition (with H_2) or by the addition of 10 mM formate. Hydrogenase and polysulfide reductase activity were measured by photometric recording of dimethylnaphthoquinone (DMN) reduction with H_2 and sulfide, respectively [2]. Formate dehydrogenase activity was measured photometrically with either benzyl viologen or DMN as acceptor as described [13]. Fumarate reductase activity was measured by photometric recording of $DMNH_2$ oxidation with fumarate as described [13]. The unit of activity (U) corresponds to conversion of 1 μmol substrate (or 2 μmol electrons) per min at 37°C.

2.5. Phospholipid

Phospholipid was determined by measuring phosphate after ashing of the sample [15]. The average molecular weight of the phospholipids was assumed to be 800.

2.6. Protein

Protein was measured using the Biuret method with KCN [16], or the method designed by Smith et al. [17] when indicated.

3. Results

3.1. Enzyme location in W. succinogenes

W. succinogenes grown with formate and polysulfide catalyzed polysulfide reduction with H_2 (Table 1). After cell disruption and fractionation of the resulting cell homogenate, the electron transport activity with H_2 (Table 1) or formate (not shown) was found exclusively in the membrane fraction. Cell disruption with the French press (Table 1) or by osmotic lysis after lysozyme treatment (not shown) caused the loss of about 70% of the electron

Table 1
Enzyme activities in the cell fractions of *W. succinogenes* grown with polysulfide

	$H_2 \rightarrow$ Polysulfide		Hydrogenase		Polysulfide reductase	
	U/mg protein	U	U/mg protein	U	U/mg protein	U
Cells	3.5	171	35	1670	30	1424
Cell homogenate	1.2	56	45	2171	30	1424
Membrane fraction	2.8	48	93	1593	69	1173
Soluble fraction	≤ 0.1	≤ 1	10	214	12	239

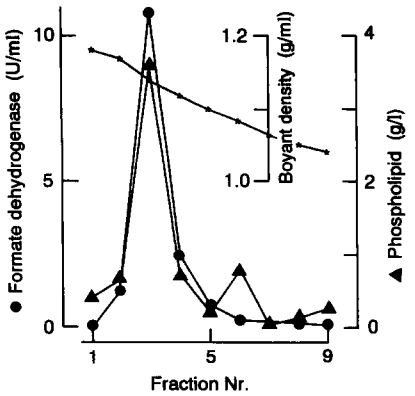

Fig. 1. Equilibrium density gradient centrifugation of fusion particles. The particles (3.2 mg phospholipid) prepared by fusion of liposomes (1 g phospholipid/g membrane protein) to the membrane fraction, were layered on top of a solution (pH 8.0, 0°C) containing 50 mM Tris chloride, 1 mM dithiothreitol, 1 mM NaN_3 and 15–60% (w/v) sucrose in a 5 ml centrifuge tube. After centrifugation for 4 h at 300000× *g* in the VTi65 rotor (Beckman), the content of the centrifuge tube was fractionated (0.5 ml per fraction). Formate dehydrogenase was measured with benzyl viologen as acceptor.

transport activities, whereas those of the dehydrogenases and polysulfide reductase were hardly affected. The electron transport activities did not decrease upon removal of the periplasmic cell fraction with lysozyme in the presence of 0.5 M sucrose (not shown). Hence, the losses in electron transport activity caused by cell disruption are not due to dilution of a periplasmic electron transport component. The actual reason is not known. It cannot be excluded that a component mediating the electron transfer from the dehydrogenases to polysulfide reductase is partially dissociated from the cytoplasmic surface of the membrane. However, incubation of the membrane fraction in buffers of high ionic strength did not cause a further loss of electron transport activity (not shown).

The enzymic activity of polysulfide reductase could only be measured using the reverse reaction with sulfide as donor and DMN as acceptor. DMN is regarded an artificial substrate of the enzyme, since a quinone is apparently not involved in the electron transport with polysulfide as acceptor, in contrast to that with fumarate. For measuring the activity of polysulfide reduction with the isolated enzyme, a sufficiently electronegative redox dye would be required. Unfortunately, reduced dyes of this type were found to react with polysulfide directly [2,4].

3.2. Fusion particles

When a mixture of the membrane fraction and liposomes (1 g phospholipid/g membrane protein) was freeze-thawed, apparently homogeneous fusion particles were obtained as shown by equilibrium density gradient centrifugation (Fig. 1). Upon fractionation of the centrifuge tube content, most of the phospholipid and formate dehydrogenase activity was found in fraction No. 3, the buoyant density (1.14 g/ml) of which corresponded to that of particles formed by complete fusion of the liposomes with the membrane fraction which contained 1.35 g phospholipid/g protein. As calculated from the proportion of total phospholipid present in fractions No. 2–4, 80% of the preparation consisted of these particles. When the membrane fraction was fused with a larger proportion of liposomes, the particles were found in the fractions of correspondingly lower buoyant density (not shown). When the experiment of Fig. 1 was done without freeze-thawing, the enzyme activity was found mainly in fraction No. 1 together with the phospholipid of the membrane fraction (not shown). The residual phospholipid was present in fraction No. 7 with a buoyant density corresponding to that of pure phospholipid (1.07 g/ml). The results suggest that freeze-thawing causes the formation of homogeneous particles from liposomal and membraneous phospholipids in which the membraneous proteins are randomly distributed. There-

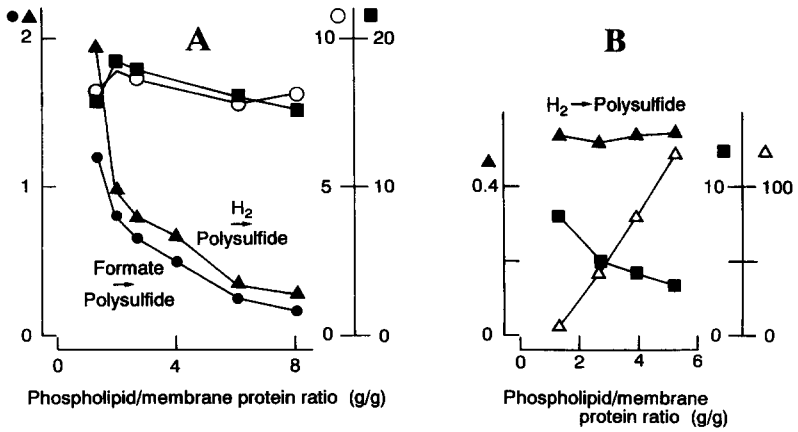

Fig. 2. Electron transport activities with polysulfide as a function of the phospholipid/membrane protein ratio of the fusion particles. The membrane fraction was prepared from *W. succinogenes* grown with polysulfide (A) or fumarate (B). Experiment (B) was done with liposomes containing hydrogenase isolated from *W. succinogenes*. The specific activities given on the ordinates in U/mg membrane protein refer to: ■, polysulfide reductase; △, hydrogenase and formate dehydrogenase (○) which was measured with DMN as acceptor. Protein was determined using the method of Smith et al. [17].

fore, the content of membrane enzymes per gram of phospholipid is assumed to decrease proportional to the amount of liposomes applied in the fusion procedure.

3.3. Electron transport activity as a function of the phospholipid / protein ratio

Increasing amounts of sonic liposomes containing vitamin K-1 were fused to a preparation of the membrane fraction of *W. succinogenes*, and the activity of the electron transport to polysulfide was measured with formate and with hydrogen as electron donor in each preparation (Fig. 2A). The specific electron transport activity based on the amount of membrane protein, decreased nearly hyperbolically as a function of the phospholipid/protein ratio of the particles. In contrast, the specific activity of polysulfide reductase and of formate dehydrogenase (Fig. 2A) as well as that of hydrogenase (not shown) remained nearly unaffected by the incorporation of phospholipid. The bacteria used in the experiment of Fig. 2A were grown with polysulfide. Similar results were obtained with fumarate-grown *W. succinogenes*, although the specific activities of polysulfide reductase and hydrogenase were considerably smaller (not shown).

In the experiment shown in Fig. 2B, the membrane fraction was fused to liposomes containing the hydrogenase isolated from *W. succinogenes* (55 mg hydrogenase protein per g phospholipid). The isolated enzyme was found to retain nearly all its activity after incorporation into liposomes and fusion of the resulting liposomal preparation to the membrane fraction. The activity of electron transport with polysulfide in the three different fusion preparations was nearly the same as that of the membrane fraction, while about half the activity of polysulfide reductase was lost in this experiment, probably due to damage of the enzyme.

The substrate sites of formate dehydrogenase and of polysulfide reductase are known to face the bacterial periplasms [3,8,18], and formate and polysulfide are unlikely to cross the membrane at a velocity commensurate to that of electron transport. Therefore, the formation of inverted vesicles would cause a decrease of the electron transport activity with the externally added substrates. The proportion of inverted vesicles in the preparations of the membrane fraction used in the experiments of Fig. 2 and Fig. 3 was measured to be less than 5% (not shown). The percentage was calculated from the activity of formate dehydrogenase before and after the addition of Triton X-100 [18]. As formate dehydrogenase activity was hardly affected by the phospholipid incorporation (Fig. 2), the proportion of inverted vesicles should be negligible also in the fusion particles. Therefore, it was unlikely that the electron transport activity with polysulfide was decreased significantly by the formation of inverted vesicles.

The formation by the fusion process of very small particles carrying only one enzyme species would also cause a decrease in electron transport activity, and should equally affect the electron transport activity with polysulfide and with fumarate as acceptor. As fumarate reduction with formate or H_2 was hardly affected by the incorporation of phospholipid (Fig. 3), the percentage of very small fusion particles appeared to be negligible.

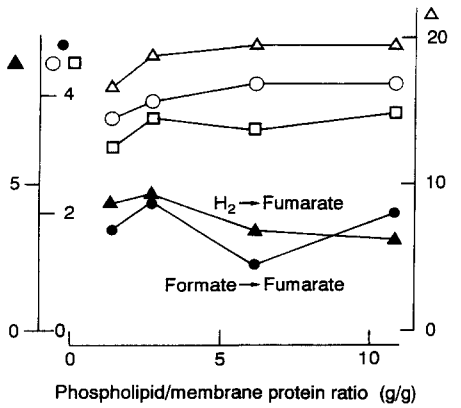

Fig. 3. Electron transport activities with fumarate as acceptor as a function of the phospholipid/protein ratio of the fusion particles. The membrane fraction was prepared from *W. succinogenes* grown with fumarate. The specific activities given on the ordinates in U/mg membrane protein refer to: △, hydrogenase; □, fumarate reductase and formate dehydrogenase (○) which was measured with DMN as acceptor. Protein was determined using the method of Smith et al. [17].

The electron transport activity to fumarate with either formate or H_2 did not decrease upon fusion to the membrane fraction of liposomes containing vitamin K-1 (Fig. 3). When the experiment of Fig. 3 was done with liposomes lacking vitamin K-1, the electron transport activity with fumarate decreased nearly proportional with the phospholipid/protein ratio (not shown). This effect can be explained by the relatively large K_m of fumarate reductase for reduced menaquinone. More than 10 μmol vitamin K-1/g phospholipid were required for saturation of the electron transport activity with liposomes containing formate dehydrogenase and fumarate reductase [14]. Vitamin K-1 is structurally very similar to menaquinone, and was found to substitute for menaquinone in bacterial electron transport [14,19].

4. Discussion

4.1. Mechanism of electron transport

The response of the electron transport activity with polysulfide to the dilution of the membrane with phospholipid argues against the existence of stable electron transport complexes. The results rather support the view that the electron transport complexes are reversibly formed from the dehydrogenase and polysulfide reductase molecules by diffusion and collision within the membrane. The specific activity of electron transport probably reflects

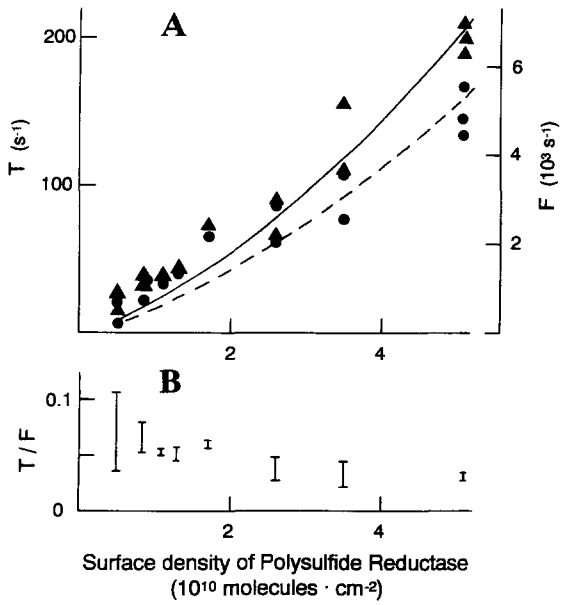

Fig. 4. (A) Turnover numbers (T, in electrons per s) of formate dehydrogenase (●) and hydrogenase (▲) in polysulfide reduction as a function of the surface density of polysulfide reductase. The data were taken from Fig. 2A and from two other equivalent experiments. T was calculated from the specific activity of electron transport with formate (●) and H_2 (▲) and the contents of formate dehydrogenase (0.25 μmol/g protein) and hydrogenase (0.30 μmol/g protein) in the membrane fraction. The surface density was calculated from the phospholipid/protein ratio and the content of polysulfide reductase in the membrane fraction (0.30 μmol/g protein). The theoretical curves were calculated according to the modified Hardt equation (1) for diffusion in two dimensions [21], where

$$F = 4\pi \cdot D \cdot S / \ln\left[(\pi S)^{-1/2}/a\right] \quad (1)$$

F represents the collision frequency of formate dehydrogenase (dashed curve) or hydrogenase (solid curve) with polysulfide reductase. The diffusion coefficient (D) of the three enzymes was assumed as 10^{-8} cm^2 s^{-1} [10]. S represents the surface density of formate dehydrogenase or hydrogenase, and a the collision radius (10^{-6} cm). (B) Ratio T/F as a function of the surface density of polysulfide reductase.

the steady state amount of the complexes, which is determined by the collision frequency of the enzymes within the membrane.

The nearly hyperbolic decrease of electron transport activity with the phospholipid/membrane protein ratio is consistent with the view that the turnover number of one enzyme in electron transport is approximately proportional to the surface density (concentration) of the other (see Fig. 4). The specific electron transport activity based on membrane protein is proportional to the turnover number of one of the enzymes in the electron transport. The phospholipid/membrane protein ratio is inversely proportional the surface density of the enzymes in the experiment of Fig. 2A. In the experiment of Fig. 2B the loss of polysulfide reductase activity is balanced by the increase in hydrogenase concentration. This explains why the activity of electron transport remains the same upon fusion of the membrane fraction with liposomes containing hydrogenase.

The results of Fig. 2 confirm the view that the MK present in the membrane of *W. succinogenes* is not involved in the electron transport with polysulfide as acceptor [2,4]. This conclusion is in line with the redox potentials. Electron transport to polysulfide ($E_0' = -0.26$ V) would require a much more electro-negative donor than menaquinone ($E_0' = -0.075$ V) [4].

4.2. Comparison of turnover numbers and diffusion velocity

In Fig. 4A the turnover number (T) of formate dehydrogenase and of hydrogenase in the electron transport with polysulfide is plotted as a function of the surface density of polysulfide reductase using the data of Fig. 2A and of two other equivalent experiments. The turnover numbers are calculated from the corresponding specific activities and the contents of the enzymes in the membrane fraction. The surface density of polysulfide reductase was calculated from its content in the membrane fraction (0.30 μmol/g protein) and the phospholipid/protein ratio, assuming that 1 g phospholipid corresponds to $2.6 \cdot 10^6$ cm^2 bilayer surface, and that the membrane proteins do not contribute to the surface of the particles [20].

The two theoretical curves drawn in Fig. 4A give the collision frequency (F) of the dehydrogenase with the polysulfide reductase molecules. The curves are calculated according to the Hardt equation [21] which relates the collision frequency of two molecules within a membrane to their diffusion coefficients (D) and their surface densities. The D values of the three *W. succinogenes* enzymes are assumed to equal that of Complex III (ubiquinone: cytochrome *c* oxidoreductase) in a preparation obtained by 7-fold dilution of the liver mitochondrial membrane with phospholipid. This value of D (10^{-8} cm^2 s^{-1}) was measured with the FRAP method (fluorescence recovery after photobleaching) [10]. Division of the measured turnover numbers (T) by the collision frequency (F) yields the theoretical proportion of productive collisions (Fig. 4B). The T/F ratios range from about 0.04 to 0.1 and decrease slightly as the surface density of the enzymes increases. This means that an electron is transferred from a dehydrogenase to a polysulfide reductase molecule after an average of 10 to 25 collisions.

The T/F ratio so obtained has to be regarded as a crude estimate. It is dependent on the validity of several assumptions under the experimental conditions used. Thus the diffusion velocity of the enzymes is assumed not to be affected by the dilution of the membrane fraction. This assumption is possibly valid only when the small diffusion distances are considered that are relevant in electron transfer. From the surface density of polysulfide reductase in the membrane fraction ($5 \cdot 10^{10}$ molecules/cm^2) (Fig. 4) the average distance between the enzymes is estimated as 40 nm. Long range (several μm) diffusion is possibly much more obstructed by the various proteins present in the membrane [22]. This may be the reason why the apparent D of Complex III measured with FRAP was

found to increase by an order of magnitude upon dilution of the mitochondrial membrane with phospholipid, and to approach the value given above at about 7-fold dilution [10]. As the diffusion distances considered with the FRAP method are in the μm range, the D values obtained at increasing dilution probably approach that valid for small distances.

The surface densities of the enzymes involved in the electron transport with fumarate as well as their turnover numbers in the electron transport are commensurate to those of the enzymes involved in polysulfide reduction. However, the activities of electron transport with fumarate do not decrease upon dilution of the enzymes with phospholipid containing vitamin K-1 (Fig. 3). This is likely to be due to the fact that the electron transfer from the dehydrogenases to fumarate reductase is mediated by menaquinone or vitamin K-1, the surface densities of which are approximately one order of magnitude larger than that of the enzymes [4,9,23]. The diffusion coefficients of menaquinone and of vitamin K-1 within the membrane should be similar to that of ubiquinone. The diffusion coefficient of ubiquinone measured by FRAP was found to be only slightly larger than that of Complex III [10], while that obtained from fluorescence quenching measurements was two orders of magnitude larger [22].

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