The Oxidative Activities of Membrane Vesicles from *Bacillus caldolyticus*

ENERGY-DEPENDENCE OF SUCCINATE OXIDATION

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1. The properties of membrane vesicles from the extreme thermophile *Bacillus caldolyticus* were investigated. 2. Vesicles prepared by exposure of spheroplasts to ultrasound contained cytochromes *a*, *b* and *c*, and at 50°C they rapidly oxidized NADH and ascorbate in the presence of tetramethyl-*p*-phenylenediamine. Succinate and L-malate were oxidized more slowly, and DL-lactate, L-alanine and glycerol 1-phosphate were not oxidized. 3. In the absence of proton-conducting uncouplers the oxidation of NADH was accompanied by a net translocation of H⁺ into the vesicles. Hydrolysis of ATP by a dicyclohexylcarbodi-imide-sensitive adenosine triphosphatase was accompanied by a similarly directed net translocation of H⁺. 4. Uncouplers (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone or valinomycin plus NH₄⁺) prevented net H⁺ translocation but stimulated ATP hydrolysis, NADH oxidation and ascorbate oxidation. The last result suggested an energy-conserving site in the respiratory chain between cytochrome *c* and oxygen. 5. Under anaerobic conditions the reduction of cytochrome *b* by ascorbate (with tetramethyl-*p*-phenylenediamine) was stimulated by ATP hydrolysis, indicating an energy-conserving site between cytochrome *b* and cytochrome *c*. However, no reduction of NAD⁺ supported by oxidation of succinate, malate or ascorbate occurred, neither did it with these substrates in the presence of ATP under anaerobic conditions, suggesting that there was no energy-conserving site between NADH and cytochrome *b*. 6. Succinate oxidation, in contrast with that of NADH and ascorbate, was strongly inhibited by uncouplers and stimulated by ATP hydrolysis. These effects were not observed when phenazine methosulphate, which transfers electrons from succinate dehydrogenase directly to oxygen, was present. It was concluded that in these vesicles the oxidation of succinate was energy-dependent and that the reoxidation of reduced succinate dehydrogenase was dependent on the outward movement of H⁺ by the protonmotive force. 7. In support of the foregoing conclusion it was shown that the reduction of fumarate by NADH was an energy-conserving process. 8. If the activities of vesicles accurately represent those of the intact organism it appears that in *B. caldolyticus* the reduction of fumarate to succinate at the expense of reducing equivalents from NADH is energetically favoured over succinate oxidation even under aerobic conditions. This may be related to the need for an ample supply of succinate for haem synthesis in order to provide cytochromes for the organism.

Kagawa and his colleagues (Yoshida *et al.*, 1975*a*; Sone *et al.*, 1975; Hirata *et al.*, 1976*a,b*) have pioneered the use of preparations from extreme thermophilic bacteria for the study of proteins of interest in the field of bioenergetics. For example, the F₁-adenosine triphosphatase (F₁, a specific polypeptide usually bound to the rest of the enzyme complex in the membrane) from the thermophile PS3 is ultra-stable and has been resolved into its component

subunits and reconstituted (Yoshida *et al.*, 1975*a,b*, 1977; Kagawa *et al.*, 1976; Sone *et al.*, 1977). In the present paper the properties of membrane vesicles from the extreme thermophile *Bacillus caldolyticus* (Heinen & Heinen, 1972), which was originally isolated from the hot springs of Yellowstone National Park, U.S.A., are described. These vesicles oxidized NADH very rapidly and succinate and L-malate at about one-sixth of the rate of NADH oxidation. However, despite the fact that uncoupling agents, e.g. carbonyl cyanide trifluoromethoxyphenylhydrazone and valinomycin together with NH₄⁺, caused a marked stimulation of both NADH oxidation and that of ascorbate in the presence

Abbreviations used: Mops, 4-morpholinopropane-sulphonic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

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of tetramethyl-*p*-phenylenediamine, they caused a marked inhibition of succinate oxidation. The oxidation of L-malate, a process that does not involve an NAD⁺-linked dehydrogenase, was not affected by uncoupling agents.

The oxidation of succinate by these vesicles results in the production of fumarate, since the particles are devoid of fumarate hydratase activity. The action of uncouplers must be therefore either on the succinate dehydrogenase or at some point in the respiratory chain used in reoxidizing the succinate dehydrogenase, but not the NADH dehydrogenase or malate dehydrogenase. It was shown that ATP hydrolysis reversed the action of low concentrations of uncoupling agents. It is suggested that succinate oxidation is dependent on the existence of a proton-motive force across the vesicular membrane. It is concluded that, for succinate oxidation to occur, energy is required in the form of a flow of H⁺ through the protonmotive force to enable the succinate dehydrogenase to reduce some more-negative component of the respiratory chains, such as low-potential cytochrome *b*.

Experimental

Culture conditions

Bacillus caldolyticus (kindly supplied by Dr. A. Atkinson of the Microbial Research Establishment, Porton, Salisbury, Wilts. SP4 0JG, U.K.) was maintained on 3% (w/v) agar plates containing 1.5% (w/v) tryptone soya broth (Oxoid, London E.C.4, U.K.) and subcultured at 1-monthly intervals. The plates, after inoculation, were placed in a polythene bag containing water at the bottom, and were incubated at 72°C for 18 h. The plates were stored at 0°C until required for use. The bacteria from one plate were used as the inoculum for liquid culture of the organism in 1.5% (w/v) tryptone soya broth. The growth vessel (total capacity 3 litres) was immersed in a water bath at 72°C and contained 1.5 litres of the culture medium, which was vigorously stirred throughout the growth period. Aerobic conditions were maintained by bubbling the culture with water-saturated air (at room temperature 18–22°C) at a rate of 5 litres/min, the exhaust gases being passed through a water-cooled reflux condenser to minimize evaporative loss. Silicone antifoaming agent (2 drops) (BDH Chemicals, Poole, Dorset, U.K.) was included in the culture medium.

Growth was followed by determining the apparent A_{620} of suitably diluted samples of the culture medium in a spectrophotometer (Cecil Instruments, Cambridge, U.K.) in a 1 cm-light-path cuvette, until the stationary phase was reached (approx. 4 h).

Harvesting and spheroplast formation

Cells were harvested by centrifugation at 3000g for 5 min and were washed once with 750 ml of 1.2%

(w/v) NaCl and once with 250 ml of 0.3 M-potassium phosphate buffer, pH 7, containing 0.5 mM-EGTA. The cells were then suspended in the potassium phosphate/EGTA buffer (1 g/10 ml), and lysozyme (Calbiochem, Los Angeles, CA, U.S.A.) (2 mg/g of cells) was added. The suspension was gently shaken at 37°C for 20–30 min, by which time the A_{620} of samples diluted 30-fold with water had fallen to less than 10% of the original value, indicating that spheroplast formation was essentially complete. Released DNA was hydrolysed by subsequent addition of 0.5 ml of 1 M-MgSO₄ and 2 mg of deoxy-ribonuclease per 100 ml of suspension and continuing the incubation at 37°C for a further 10 min. The spheroplasts were sedimented by centrifugation at 17000g for 10 min.

Preparation of membrane vesicles

Spheroplasts were suspended (1 g/5 ml) in 0.3 M-potassium phosphate buffer, pH 7, containing 5 mM-MgSO₄, 0.5 mM-EGTA and 0.002% (w/v) deoxy-ribonuclease. The suspension was exposed to ultrasound for 1 min at 16 kHz (power output 80–100 W) with a Dawe Soniprobe type 7532 A (Dawe Instruments, Acton, London, U.K.). To isolate the vesicles the sonicated suspension was first centrifuged at 10000g for 10 min to remove unbroken cells and spheroplasts together with other dense material. The supernatant was then centrifuged at 40000g for 30 min in an MSE High-Speed 18 centrifuge, and the resultant pellet of membrane vesicles was resuspended in 0.3 M-K₂SO₄ containing 5 mM-MgSO₄, 5 mM-Mops and 2.5 mM-Tris/HCl (pH 7.3) before re-centrifuging at 40000g for 30 min. The final pellet of washed vesicles was suspended (1 g/10 ml) in the buffered K₂SO₄/MgSO₄ medium and contained approx. 75% of the NADH oxidase activity of the original sonicated suspension. The vesicle suspension could be stored for at least 2 weeks at 4°C without any detectable loss of respiratory activity and uncoupler sensitivity.

Incubations and measurements

Experiments were performed as a routine at 50°C and pH 7.6. In most cases a medium comprising 0.3 M-K₂SO₄ and 5 mM-MgSO₄ buffered with 5 mM-Mops and 2.5 mM-Tris was used for incubations; any alterations to this medium are described in the legends to the relevant Figures. Vesicles were suspended in 2–3 ml of the incubation medium either in the reaction chamber of a Rank oxygen electrode (Rank Bros., Bottisham, Cambridge, U.K.) which was also fitted with a pH electrode, or in a sealed quartz cuvette, depending on the type of experiment. Substrates, uncouplers and other agents were added in the smallest volumes feasible.

The medium was previously bubbled with CO₂-free water-saturated air (at the temperature of the

experiment). The concentration of O_2 in the media at different temperatures was determined by recording the amount of oxygen consumed on addition of known amounts of NADH to a vesicle suspension, as described by Chappell (1964). At 50°C for the medium described in the present paper the solubility was 0.41 $\mu\text{g-atom of O/ml}$.

ATP hydrolysis was determined by measuring the changes in pH resulting from the dissociation of the acid products of hydrolysis as described by Chance & Nishimura (1967).

The oxidation of NADH by fumarate in the absence of oxygen was measured by following the decrease in A_{340} corresponding to NADH. Anoxic conditions were achieved by bubbling the reaction mixture with O_2 -free N_2 and then adding NADH to remove the final traces of O_2 .

Succinate dehydrogenase activity was assayed by following the reduction of oxygen by succinate in the presence of 0.8 mM-phenazine methosulphate, which transfers electrons directly from the flavin group of succinate dehydrogenase to oxygen (Singer, 1966).

Spectrophotometric observations

These were made on an instrument constructed in the Mechanical Workshops of the Medical School of this University, by using the principles outlined by Chance (1957). The spectrophotometer was used to determine difference spectra in one mode and the kinetic changes in cytochrome redox state in the dual-wavelength (double-beam) mode. For recording difference spectra the vesicle preparation was contained in two separate quartz cuvettes of 1 cm light-path; the cell housing was thermostatically controlled at 50°C. For dual-wavelength measurements the vesicle suspension was contained in a cuvette of special design. This consisted of a temperature-controlled titanium metal block in which suitable holes had been bored to allow the insertion of a stirrer at the bottom and three quartz windows for optical measurements; two of these were in the optical axis for absorption measurement, and the third was at a right-angle (this was used for fluorescence measurements). The fourth side of the cuvette contained a Clark-type oxygen electrode. The top of the cuvette was sealed with a tight-fitting Teflon plug, through which was drilled a narrow channel and through which additions could be made with a micro-syringe.

Fluorescence was measured in the cuvette described above. Excitation was provided by the modulated light from one of the two monochromators. Fluorescence was recorded at right-angles. The recording photomultiplier had a suitable combination of filters over its front face. The fluorescence of 9-aminoacridine was elicited by excitation at 425 nm

and that of atabrin at 430 nm; in both cases the secondary filter was a Corning glass filter 4015. The fluorescences of 8-anilino-naphthalene-1-sulphonate and of 2-*p*-toluidinylnaphthalene-6-sulphonate were elicited at 365 nm and recorded with the same secondary filter.

Assay of protein

This was determined by the biuret method (Jacobs *et al.*, 1956), with bovine plasma albumin as standard.

Materials

All materials were of A.R. grade where available. Carbonyl cyanide trifluoromethoxyphenylhydrazone, valinomycin and fumarate hydratase were supplied by Boehringer, London W5 2TZ, U.K. Atabrin, 9-aminoacridine and naphthalenesulphonate dyes were purchased from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.

Results

*Oxidative activities of vesicles from *B. caldolyticus**

NADH, succinate, L-malate and ascorbate in the presence of tetramethyl-*p*-phenylenediamine were oxidized by these vesicles (Table 1). No measurable oxygen consumption was observed in the presence of 5 mM-L-alanine, fumarate, DL-glycerol 1-phosphate, D- or L-lactate, D-malate or oxaloacetate, under the conditions of these experiments.

L-Malate was quantitatively oxidized, with a malate/O ratio of 1. The low apparent K_m for malate (approx. 100–150 μM) enabled the oxygen consumption corresponding to the addition of known amounts of malate to be determined with accuracy. Further, oxaloacetate was not oxidized by these particles.

Table 1. *Oxidation of various substrates*

Vesicles (0.15–0.3 mg of protein) were incubated at 50°C in 2 ml of the buffered $K_2SO_4/MgSO_4$ medium (see the Experimental section) in the presence of NADH, succinate, L-malate or ascorbate with tetramethyl-*p*-phenylenediamine. Results are expressed as mean values $\pm$ S.E.M. with the numbers of separate preparations in parentheses.

Substrate	Oxygen uptake ($\mu\text{g-atoms of O/min}$ per mg of protein)
0.7 mM-NADH	1.37 ± 0.14 (6)
5 mM-Succinate	0.28 ± 0.05 (5)
5 mM-L-Malate	0.23 ± 0.04 (4)
2.5 mM-Ascorbate + 0.25 mM-tetra- methyl- <i>p</i> -phenylenediamine	1.16 ± 0.20 (3)

Succinate was quantitatively converted into fumarate. This was demonstrated as follows: 5 mM-succinate was oxidized by the particles until approx. 40% of the oxygen present had been consumed; 5 μ M-FCCP was then added, which caused an abrupt and complete inhibition of further succinate oxidation. Then fumarate hydratase (0.05 mg of protein) was added and the resulting O₂ consumption was recorded. The results were consistent with the following reactions occurring:

It may be concluded therefore that these particles catalyse a one-step oxidation of succinate to fumarate and of malate to oxaloacetate, or perhaps pyruvate.

Sidedness of the vesicle preparation

Untreated spheroplasts of *B. caldolyticus* showed little capacity for the oxidation of exogenous NADH or succinate. In contrast, sonicated spheroplasts showed an increased NADH and succinate oxidation, which was respectively enhanced and inhibited by uncoupling agents and an unchanged capacity compared with untreated spheroplasts for the oxidation of ascorbate together with tetramethyl-*p*-phenylenediamine. These observations are consistent with the existence of a permeability barrier to NADH and succinate in intact spheroplasts and the exposure to the external media of the dehydrogenase active sites on sonication.

The addition of ATP or of an oxidizable substrate to vesicles resulted in an acidification of the interior of the vesicles, as revealed by the quenching of the fluorescence of 9-aminoacridine or atabrin (Schuldinger *et al.*, 1972; Deamer *et al.*, 1972) (Fig. 1). In a 0.3 M-choline chloride medium, the internal acidification did not occur to any appreciable extent in the

Fig. 1. Simultaneous recordings of fluorescence changes of atabrin and oxygen consumption by membrane vesicles of *B. caldolyticus*

Initially membrane vesicles (1.1 mg of protein) were added to 2 ml of medium containing 10 μ M-atabrin, 5 mM-Mops, 2.5 mM-Tris and (a) 0.3 M-choline chloride or (b) and (c) 0.3 M-KCl. The final pH was 7.4 at 50°C. Where indicated 0.21 mM-NADH, 1 μ M-FCCP and 2 μ M-valinomycin (final concns.) were added. Fluorescence; —, O₂ consumption. The curve labelled (c) is the latter part of a trace which was initially identical with that shown in (b) to the point where valinomycin was added.

Fig. 2. Oxygen uptake by vesicles oxidizing NADH and succinate

Vesicles were incubated at 50°C in 2 ml of the buffered K_2SO_4 medium (see the Experimental section). Additions were made at the points indicated by arrows. The numbers alongside the curves give the rate of O_2 consumption in $\mu\text{g-atoms of O/min per mg}$ of protein. The curves were traced from an actual recording.

presence of $1 \mu\text{M-FCCP}$ or of valinomycin ($1 \mu\text{g}/2 \text{ ml}$) together with $10 \text{ mM-NH}_4\text{Cl}$. In a 0.3 M-KCl medium, the addition of valinomycin alone very largely suppressed the internal acidification. These findings are consistent with the existence of an inwardly directed H^+ pump, but do not preclude the possibility that a proportion of the vesicles have the opposite polarity or are ruptured.

By using either 8-anilinoanthracene-1-sulphonate or 2-*p*-toluidinylnaphthalene-6-sulphonate, it was shown that the oxidation of succinate or malate or the hydrolysis of ATP led to a marked increase in fluorescence of either of the probes. Uncoupling agents or, with oxidizable substrates, anaerobiosis led to a reversal of the fluorescent changes. It is commonly held that increased anilinoanthracene- and toluidinylnaphthalene-sulphonate fluorescences reflect the existence of a membrane potential positive inside (Azzi *et al.*, 1969).

These findings taken together indicate that most, if not all, of the vesicles are orientated in such a way that H^+ ions are pumped inwards, with the consequent generation of a membrane potential positive inside. The relative impermeability of the vesicles to H^+ , revealed by the stimulation of NADH and ascorbate oxidation by uncouplers, precludes the

Fig. 3. Effects of FCCP and ATP on oxygen uptake by vesicles oxidizing succinate

Vesicles (0.32 mg of protein) were incubated at 50°C in 2 ml of 0.5 M-sucrose containing 5 mM-MgSO_4 and 0.1 mM-EGTA , buffered to $\text{pH} 7.6$ with $10 \text{ mM-Tris/sulphate}$; 5 mM succinate was present in all systems. FCCP and ($\circ$) no or ($\bullet$) 5 mM-ATP were added as shown.

possibility that the substrates first penetrate the vesicles before they are oxidized, since it is unlikely that a vesicle would be impermeable to H^+ yet permeable to NADH, succinate or malate.

Effects of uncouplers and ATP on the oxidation of succinate, NADH and ascorbate

Fig. 1 shows that $10 \mu\text{M-FCCP}$ markedly stimulated the oxidation of NADH by the vesicles; in other experiments (results not shown) a twofold stimulation of ascorbate oxidation in the presence of tetramethyl-*p*-phenylenediamine was observed. In contrast, the oxidation of succinate was strongly inhibited even by $1 \mu\text{M-FCCP}$, an effect that was completely reversed by the addition of ATP (Fig. 2). That the inhibition of succinate oxidation by FCCP was due to the uncoupling action of the latter compound was demonstrated by experiments in which other uncouplers, notably valinomycin together with $(\text{NH}_4)_2\text{SO}_4$, were shown to have the same effect as FCCP (see Fig. 5). However, the ability of ATP to overcome the inhibition depended on the concentration of the uncoupler, and Fig. 3 shows that, although succinate oxidation was less susceptible to inhibition by FCCP in the presence of ATP than in its absence, ATP was unable fully to prevent inhibition when the uncoupler concentration exceeded $5 \mu\text{M}$.

The conclusion drawn from the above results was that, when the vesicles were made freely permeable to H^+ , their ability to oxidize NADH and ascorbate was increased, but their ability to oxidize succinate was lost. This conclusion was supported by an

Fig. 4. Effect of dicyclohexylcarbodi-imide on succinate oxidation and ATP hydrolysis

Vesicles (0.55 mg of protein) were preincubated for 10 min at 50°C in 3 ml of the buffered $\text{K}_2\text{SO}_4/\text{MgSO}_4$ medium (see the Experimental section) containing dicyclohexylcarbodi-imide at the concentration indicated. Additions were then made as follows: 5 mM-succinate (at zero time), 1 μM -FCCP (at 1 min), 1 mM-ATP (at 2 min). Oxygen consumption and pH changes were monitored continuously; the curves in the Figure represent O_2 consumption in the absence of FCCP and ATP (●), O_2 consumption in the presence of FCCP (△), O_2 consumption in the presence of FCCP and ATP (○), H^+ production in the presence of FCCP and ATP (□).

analysis of experimental data on 11 separate preparations of vesicles showing that a rapid rate of succinate oxidation occurred only in those preparations in which NADH oxidation was strongly stimulated by FCCP, i.e. in vesicles that were relatively impermeable to H^+ .

Further investigations revealed that the restoration of succinate oxidation by ATP was brought about through ATP hydrolysis and not by any direct regulatory action of the nucleotide itself. Fig. 4 shows that when ATP hydrolysis was inhibited by dicyclohexylcarbodi-imide there was a corresponding decrease in the ability of added ATP to reverse the inhibition of succinate by FCCP, though dicyclohexylcarbodi-imide did not influence succinate oxidation in the absence of FCCP and ATP. This result strongly suggested a causal relationship between ATP hydrolysis and increased succinate oxidation, a view that was strengthened by the obser-

Fig. 5. Effect of ATP hydrolysis on succinate oxidation in the presence of uncoupler

Vesicles were incubated at 50°C in 3 ml of 0.1 M- K_2SO_4 containing 0.3 mM- $(\text{NH}_4)_2\text{SO}_4$ and buffered to pH 7.6 with 5 mM-Mops and 2.5 mM-Tris. Succinate, valinomycin and Mg-ATP were added at the points marked by arrows. The curves were traced from an actual recording.

Fig. 6. Effect of FCCP on ATP hydrolysis and ATP-stimulated succinate oxidation

Vesicles (0.22 mg of protein) were incubated in 3 ml of the buffered $\text{K}_2\text{SO}_4/\text{MgSO}_4$ medium (see the Experimental section). Succinate, ATP and FCCP were added at the points indicated by arrows. The curves were traced from an actual recording.

vation that in vesicles oxidizing succinate in the presence of low concentrations of uncoupler [FCCP or valinomycin together with $(\text{NH}_4)_2\text{SO}_4$] the increase in oxygen consumption after ATP addition followed precisely the same time-course as ATP hydrolysis (Fig. 5). Since neither ADP nor P_i , the products of ATP hydrolysis, had any detectable effect on succinate oxidation (results not shown), it was inferred from the above experiments that the promotion of succinate oxidation by ATP was due to an energetic change in the membrane vesicles brought about by ATP hydrolysis. Further evidence for this was provided by the results given in Fig. 6 showing that, when ATP stimulated succinate oxidation in the absence of uncoupler, the increase in oxygen consumption was again temporally associated with ATP hydrolysis. Subsequent addition of FCCP stimulated ATP hydrolysis, but inhibited succinate oxidation; this indicated that even increased ATP hydrolysis could not sustain the maximum rate of succinate oxidation when the permeability of the membrane vesicles to H^+ was increased.

Effect of NADH oxidation on succinate oxidation

If the above interpretations are correct, it might be predicted that the creation of a protonmotive force by mechanisms other than ATP hydrolysis should also stimulate succinate oxidation. The results in Fig. 7 support this prediction in that they show a 50% increase in the rate of succinate oxidation after the oxidation of a small amount of NADH. A further

Fig. 7. *Effect of NADH on succinate oxidation*
Each system contained 0.15mg of vesicle protein.
Other experimental conditions were as for Fig. 1.
The curves were traced from actual recordings.

increase was elicited by the subsequent addition of ATP. NADH did not stimulate succinate oxidation in the presence of ATP, indicating that ATP hydrolysis alone energized the vesicles sufficiently for maximum succinate oxidation to occur.

Lack of effect of FCCP and ATP on succinate dehydrogenase activity

The rate at which succinate dehydrogenase removed electrons from succinate was determined by measuring the rate of oxygen consumption in the presence of phenazine methosulphate. This was approximately three times the rate at which succinate

Fig. 8. *Oxidation of NADH by fumarate in the absence of oxygen*

Vesicles (0.44mg of protein) were incubated at 50°C in 3ml of oxygen-free buffered $\text{K}_2\text{SO}_4/\text{MgSO}_4$ medium containing 0.2mM-NADH (see the Experimental section). Fumarate, FCCP and ATP were added at the points indicated. The numbers alongside the curve give the rate of NADH oxidation in $\mu\text{mol}/\text{min}$ per mg of protein.

Fig. 9. Changes in the redox state of *b* cytochromes followed by dual-wavelength spectrophotometry at 562–575 nm. An upward deflexion corresponds to cytochrome *b* reduction. Vesicles (0.6 mg of protein/ml) were suspended in 2 ml of 0.3 M-KCl containing 5 mM-MgSO₄, 5 mM-Mops and 2.5 mM-Tris. The pH was 7.4 at 50°C. The experiments started by the addition of 1.5 μmol of NADH (not shown in trace *b*). After approx. 1 min the suspension became anaerobic; then 1 μM-FCCP and 5 mM-fumarate were added as shown. At each of these additions a small amount of O₂ was added; this was utilized and a new steady state was established.

reduced oxygen in the absence of phenazine methosulphate and it was not significantly affected by either ATP or FCCP. The effects of these substances on succinate oxidation must therefore be exerted on some step in the oxidative pathway after succinate dehydrogenase.

Reduction of fumarate by NADH in the absence of oxygen

If, as indicated by the above results, succinate oxidation is promoted by an increase in the protonmotive force across the vesicle membrane, the reverse process, reduction of fumarate, should, if it is achieved by a reversal of the pathway, be promoted when the protonmotive force is dissipated. Fig. 8 shows that the oxidation of NADH in the absence of oxygen was stimulated by fumarate, though the rate of NADH oxidation was much slower than it was in the presence of oxygen. Oxidation of NADH by fumarate was stimulated by FCCP and inhibited by ATP, responses to those of succinate oxidation.

Since it was not possible to demonstrate ATP-dependent reduction of NAD⁺ by succinate in the absence of oxygen, an observation that suggests that there is no energy-conserving step in that region of the respiratory chain between NADH and the point of entry of electrons from succinate, it was concluded that the FCCP-sensitive step in the reduction of fumarate by NADH lay between the main respiratory chain and succinate dehydrogenase.

Support for this viewpoint was provided by spectrophotometric experiments of the type shown in Fig. 9. NADH caused an extensive reduction of cytochromes *b* under anaerobic conditions; the further addition of fumarate caused an oxidation of cytochrome *b*, which was even more marked in the presence of FCCP. FCCP alone did not affect the extent of reduction of cytochrome *b* in the absence of fumarate. Had there been a rate-controlling step limited by the protonmotive force lying between NADH and the *b* cytochromes, it would have been expected that FCCP would have caused a further reduction in the presence of fumarate.

Cytochromes b of B. caldolyticus vesicles

In Fig. 10 difference spectra in the region 540–580 nm for the *b* and *c* cytochromes of vesicles are shown. Ascorbate together with tetramethyl-*p*-phenylenediamine produced a spectrum typical of the α -bands of cytochromes *c*; the maximum was at 552 nm and may well be compounded of a *c* and a *c*₁ cytochrome. When both the experimental and reference cuvettes contained ascorbate together with tetramethyl-*p*-phenylenediamine under anaerobic conditions and NADH was added to the experimental cuvette, a typical *b*-type spectrum resulted, with a maximum at 564 nm. When succinate was added, rather than NADH, a smaller peak with the same maximum resulted.

Fig. 10. Difference spectra for *B. caldolyticus* vesicles in the cytochrome *b* and *c* α -band region

Vesicles (3.1 mg of protein/ml) were suspended in 0.3 M-KCl/5 mM-Mops/2.5 mM-Tris/1 mM-MgSO₄; the final pH was 7.4 at 50°C. In (a) the baseline was first recorded (oxidized-oxidized; horizontal curve, ———), after which 2.5 mM-ascorbate together with 0.25 mM-tetramethyl-*p*-phenylenediamine (final concns.) were added to the experimental cuvette. The spectrum was recorded after the establishment of anaerobic conditions (—). Then 5 mM-ATP was added to the experimental cuvette and after the few seconds required for anaerobiosis the spectrum was re-recorded (----). In (b) 2.5 mM-ascorbate together with 0.25 mM-tetramethyl-*p*-phenyldiamine was added to both experimental and reference cuvettes and after anaerobiosis the horizontal curve (——) was recorded (reduced-reduced). Then either 0.5 mM-NADH (lower curve) or 5 mM-succinate (upper curve) (final concns.) was added and after anaerobiosis the spectra were recorded.

The *b* cytochromes could also be reduced by ascorbate together with tetramethyl-*p*-phenylenediamine in the presence of ATP (Fig. 10), a process that was inhibited by the addition of dicyclohexylcarbodi-imide. This represents an energy-dependent reduction of cytochromes *b* by cytochromes *c* and reflects the presence of an energy-conservation site in that region of the respiratory chain.

Discussion

Energy-conservation sites in *B. caldolyticus* vesicles

Scheme 1 shows the regions of the respiratory chain of this bacterium in which it is thought that an energy conservation may occur. That there is an energy-conservation site between cytochrome *c* and oxygen is indicated by the finding that the oxidation of ascorbate together with tetramethyl-*p*-phenylenediamine is stimulated by uncoupling agents (FCCP or valinomycin together with NH₄⁺) and that ascorbate oxidation results in reversed electron transport with the consequent reduction of cytochrome *b*. This latter observation, together with the finding that there was a dicyclohexylcarbodi-imide-sensitive ATP-driven reduction of *b* cytochromes in the presence of ascorbate together with tetramethyl-*p*-phenylenediamine under anaerobic conditions, is

good evidence for an energy-conservation site between cytochromes *b* and *c*.

No evidence could be obtained for an energy-conservation site between NADH dehydrogenase and cytochromes *b*. Thus, despite exhaustive efforts, no energy-linked reduction of NAD⁺ supported by oxidation of succinate, malate or ascorbate (+ tetramethyl-*p*-phenylenediamine) was demonstrable. Under similar conditions with *Escherichia coli* preparations, Sweetman & Griffiths (1971) and Poole & Haddock (1974) have been able to demonstrate energy-linked NAD⁺ reduction. It appears therefore that in these vesicles there is no energy conservation associated with the passage of reducing equivalents from NADH to cytochrome *b*, which may reflect the situation in the native *B. caldolyticus* organism.

The observations that succinate oxidation is inhibited by uncoupling agents (FCCP or valinomycin together with NH₄⁺), and that at sufficiently low FCCP concentrations the inhibition can be reversed by the addition of ATP and that this ATP effect is in turn prevented by dicyclohexylcarbodi-imide (Figs. 2 and 3), are all consistent with the view that succinate oxidation occurs only when the vesicles have a protonmotive force across their membranes, that is the vesicles are 'energized'. It seems likely that this energy requirement is at a

site between the succinate dehydrogenase and at least one of the *b* cytochromes. Thus when phenazine methosulphate acted as electron acceptor, presumably mainly from the flavin of the succinate dehydrogenase, FCCP or ATP was without significant effect. In contrast, the flow of reducing power from NADH to fumarate under anaerobic conditions was enhanced by FCCP and decreased by ATP (Fig. 8), behaviour that was reflected in the steady-state reduction of the *b* cytochromes (Fig. 9). If it is accepted that there is no energy-conservation site between NADH and the *b* cytochromes, then the only

interpretation of these findings is that the flow of reducing power from *b* cytochromes to fumarate is associated with energy conservation, and the reverse process, from succinate to cytochrome *b*, is associated with energy utilization.

Biological significance of these findings

The consequence of the existence of an arrangement such as that shown in Scheme 1 is that in a system in which NADH oxidation is occurring the succinate-fumarate couple is driven to a more negative potential than it would be if the succinate dehydrogenase interacted with the *b* cytochromes without an energy-requiring step. This means that succinate synthesis is favoured, rather than succinate oxidation; there is an energy-conserving reduction of fumarate to succinate at the expense of reducing equivalents provided by the oxidation of NADH. In an organism with a high oxidative capacity this may be of importance, since succinate is a precursor of haem synthesis, and large quantities of haem would be required for cytochrome synthesis in this rapidly growing bacterium.

An analogous energy-dependent oxidative system occurs in *Nitrobacter*; the oxidation of NO_2^- by this organism with O_2 as acceptor is inhibited by uncoupling agents. In this case the energy-dependent step appears to be the reduction of cytochrome *c*-551 by NO_2^- (Kiesow, 1967; Cobley, 1973).

Scheme 1. Sites of energy conservation or utilization in the oxidation of substrates by *B. caldolyticus* vesicles

A dashed arrow pointing upwards represents H^+ movement into the vesicle. See the text for discussion. TMPD, Tetramethylphenylenediamine.

Scheme 2. Representation of Mitchell's (1977) 'Q-cycle' for the case of *B. caldolyticus*

Q is a quinone, which in this organism is probably menaquinone (J. Beavis, personal communication). $\text{QH}\cdot$ is the semiquinone. The cycle is shown for the passage of one reducing equivalent from NADH or malate to oxygen through the respiratory chain; this results in (a) the injection of 2H^+ and (b) two electrophoretic steps [cytochrome b_1 to b_h (low potential and high potential respectively) and c_1 to a_3] in which cytochromes cause an outward transport of electrons. There appears to be no way in which this cycle could be adapted to accommodate the finding with succinate described in the present paper.

Relationship to chemiosmosis

It is now commonly accepted that vectorial charge displacement of H^+ in one direction and of negatively charged entities in the other direction, resulting in the production of a protonmotive force, is a fundamental mechanism of energy conservation in biology (Mitchell, 1968; Harold, 1977). The protonmotive force may be utilized in a number of different ways, including the synthesis of ATP, reversed electron transport, energy-linked transhydrogenase and bacterial motility. The oxidation of succinate by *B. caldolyticus* vesicles belongs to this class of processes and is perhaps most directly analogous to the energy-linked transhydrogenase.

More recently Mitchell (1977) has proposed the so-called 'Q-cycle' to account for the mechanism of H^+ ejection associated with respiratory-chain systems. A version of this, adapted for the present discussion, is shown in Scheme 2; since it appears that there is no energy conservation associated with the NADH dehydrogenase part of the respiratory chain in this organism, the NADH dehydrogenase and malate dehydrogenase are shown feeding in reducing power at the points shown. However, it does not appear possible to us to accommodate in this scheme the fact that reduction of cytochrome *b* by succinate is an energy-dependent process.

An alternative to account for H^+ ejection by respiratory-chain systems is the conformational hypothesis or vectorial Bohr-shift mechanism [see Mitchell (1977) for references]. In the present context, the part of the respiratory chain from cytochromes *b* to *c*, and from cytochrome *c* to oxygen, would be regarded as H^+ pumps, causing the movement of H^+ into the vesicles. In this case the succinate dehydrogenase system would be required to undergo a conformational change, associated with the outward movement of H^+ , before it could react with the rest of the respiratory chain.

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