Respiration-driven Proton Transport in Submitochondrial Particles

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SUMMARY

1. The maximal H+:O ratios of proton transport in highly resolved submitochondrial particles prepared by sonication of bovine heart mitochondria in the presence of ammonia and exposed sequentially to Sephadex and urea (ASU particles) in the presence of oligomycin were found to be 6, 4, and 2 during the oxidation of NADH, succinate, or N-methylphenazonium methosulfate, respectively. A pH dependence for the H+:O ratio was observed with optimal ratios at pH 6.0 to 6.5.

2. Comparison of the effects of an uncoupler (p-trifluoromethoxy carbonyl cyanide phenylhydrazone) and of oligomycin on proton transport and on respiratory control in ASU particles showed that the effect of these compounds on the respiratory rate could be accounted for by their effect on the proton permeability of the particles.

3. The external and internal buffering powers of ASU particles were calculated from the extent of "overshoot" of pH change on addition of acid or alkali.

4. Coupling factors of oxidative phosphorylation, F1 and oligomycin sensitivity-conferring protein, decreased the proton permeability of ASU particles and allowed proton transport to be observed in the absence of oligomycin.

There is considerable evidence that proton transport is associated with reactions of oxidative phosphorylation and that transport of other ions and acids in mitochondria is driven by the membrane potential and pH gradient created by proton transport (1-5). The chemiosmotic hypothesis of oxidative phosphorylation proposes that proton transport, in addition to driving transport of other ions, is the process common to the oxidation and phosphorylation reactions which effects coupling between them (4, 5).

Mitchell and Moyle (6) reported that proton transport driven by respiration in submitochondrial particles made by sonic disintegration of mitochondria is directed inward, in contrast to mitochondria where it is directed outward. This is consistent with other evidence that submitochondrial particles are inverted with respect to mitochondria (7). The inversion of the inner mitochondrial membrane exposes NADH and succinate dehydrogenases and the ATPase (F1) to the suspending medium, and thus allows oxidation and phosphorylation reactions to occur without the need for transport of substrates and adenine nucleotides across the membrane. Submitochondrial particles have also been resolved into soluble protein coupling factors required for phosphorylation in deficient particles such as ASU particles (8, 9). It is of interest that coupling factors, as well as the inhibitor oligomycin, induce respiratory control released by uncoupling agents in ASU particles and also stimulate energy-linked reactions in addition to phosphorylation (7, 10-12). This coupling effect of oligomycin has been proposed to result from the inhibition of some energy-dissipating process, such as the hydrolysis of an intermediate compound (7) or a decrease in proton permeability of the membrane preventing dissipation of the electrochemical gradient of protons formed during respiration (5).

This paper describes some properties of respiration-driven proton transport in submitochondrial particles and the effects of oligomycin and coupling factors on this transport process.

MATERIALS AND METHODS

Reagents—Oligomycin, alcohol dehydrogenase, and catalase were purchased from Sigma. Carbonyl cyanide p-trifluoromethoxyphenylhydrazone was generously donated by Dr. P. G. Heytler of du Pont. Valinomycin was a gift from Dr. B. C. Pressman.

Preparations—ASU particles (9), F1 (13), and purified OSCP (14, 15) were prepared as previously described. Particle protein was determined by the biuret procedure in the presence of 0.33% deoxycholate (16).

Measurements of pH—The pH was continuously measured with a Beckman glass electrode No. 39045, Vibron electrometer 23 B-2, pH measuring unit C32 B-2 (Cambridge Instrument Company) and recorder (Heath Company).

A closed glass reaction vessel was constructed similar to that described by Mitchell and Moyle (17). A stirrer was made from a 0.25-ml glass syringe as previously described (18). Additions were made to the vessel with Hamilton syringes. The vessel

1 The abbreviations used are: F1, coupling Factor 1 (ATPase); ASU particles, submitochondrial particles prepared by sonication of bovine heart mitochondria in the presence of ammonia and exposed sequentially to Sephadex and urea; OSCP, oligomycin sensitivity-conferring protein, a coupling factor; FMS, N-methylphenazonium methosulfate; FCCP, p-trifluoromethoxy carbonyl cyanide phenylhydrazone.
and the reference calomel electrode were maintained at 25°C by water circulated from a constant temperature water bath. The shield covering the lead from the glass electrode was extended with aluminum foil around the ground glass joint holding the electrode to prevent electrostatic interference. This apparatus allowed the pH of the suspension medium to be recorded at 0.1 pH unit full scale with very low drift or noise and with rapid stirring so that relatively rapid (1 sec) changes in pH could be followed. The volume of the vessel was 1.05 ml.

Various media were made anaerobic by bubbling with nitrogen in a 30-ml syringe for 10 min and were dispensed from the syringe into the reaction vessel without coming in contact with air. Small additions to the vessel were made with 10-μl Hamilton syringes. The button on the wire plunger of the syringe was covered with parafilm to prevent electrical contact with the medium when making an addition. Buffering power of the suspension was measured by adding small amounts of anaerobic 10 mM HCl.

Measurement of Respiration—Respiration was measured in a separate apparatus with a Clark oxygen electrode.

RESULTS

Efficiency of Proton Transport—The conditions for observing proton transport in submitochondrial particles are well established (6, 10, 11, 19-21). As in mitochondria, a permeant coion such as valinomycin-potassium, nitrate, thiocyanate, tetraphenylboron, or other organic ions is required for a large amount of proton uptake. In coupling factor-deficient particles oligomycin is also required (10). The efficiency of respiration-driven proton transport (H+/O) was measured by the oxygen pulse technique of Mitchell and Moyle (1). Particles (0.5 to 5 mg of protein) were added to 1 ml of anaerobic medium containing 150 mM KCl, 3 mM glycylglycine, and substrate. Oligomycin and valinomycin were added, and the system was allowed to equilibrate for at least 3 min. During this equilibration of the internal and external potassium concentrations the pH decreased, indicating proton efflux driven by potassium ion influx. Fig. 1A shows a recording of pH during an oxygen pulse experiment with succinate as substrate. Addition of 10 μl of air-equilibrated 150 mM KCl to the system caused a rapid increase in pH, indicating proton uptake (or hydroxide ion extrusion) by the ASU particles. The slow return of the pH to the original value after respiration has ceased may be taken as a measure of the proton permeability of the membrane as protons diffuse out of the particles. When valinomycin was omitted some proton transport was observed which was variable depending on the particles and the KCl concentration of the medium (not shown). To calculate the H+/O ratio the extent of proton uptake at 0.1 sec intervals following an oxygen pulse was plotted on a logarithmic scale (Fig. 1B) and the first order decay curve was extrapolated to the point when respiration ceased, about 1 sec after addition of the oxygen. This extrapolation corrects for the inability of the pH electrode to follow the rapid change in pH at the beginning of the oxygen pulse. Fig. 2 shows a summary of H+/O ratios determined with ASU particles at several pH values and with different substrates. In the region of pH from 6.0 to 6.5 the H+/O ratios were close to 6, 4, and 2 with ethanol plus NAD, succinate, and reduced 1,4-naphthoquinone-2-sulfonate plus PMS, respectively, as substrates. These values are in agreement with the reported values of 6 and 4 with hydroxybutyrate and succinate, respectively, in rat liver mitochondria (1). At higher pH values, however, the H+/O ratios with all three substrates declined. The reason for this pH dependence of the H+/O ratio has not been determined, but one possible explanation is indicated by the experiment shown in Fig. 3. When low levels of the uncoupling agent FCCP are added, the diffusion of protons from the particles after respiration ceases is more rapid (Fig.
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Fig. 3. Effect of FCCP and NH₄Cl on measurement of H⁺:O ratio. The system contained 150 mKNO₃, 5 mM glycylglycine (pH 6.5), 2 mg of ASU particles, 3 µg of oligomycin, and 2 mM succinate in a final volume of 1.05 ml. A, 4.8 ng atoms of oxygen added in the presence of several concentrations of FCCP as labeled. B, 4.8 ng atoms of oxygen added in the presence of several concentrations of NH₄Cl as labeled.

Fig. 4. Effect of oligomycin on respiratory rate and t₁/₂ of pH equilibration in ASU particles. ASU particles were treated with different amounts of oligomycin and aliquots were taken for the determination of respiration rate and for the measurement of proton transport with NADH as substrate as described in Figs. 1 and 2 and in the text.

3A). When the correction for the slow response of the pH electrode is made, however, the H⁺:O ratio is unchanged by the uncoupler, as reported by Mitchell and Moyle (1). Thus the oxygen pulse technique is a valid measure of the H⁺:O ratio even when the particles are partially uncoupled. If low levels of a permeant base such as ammonia are added, however, the rate of relaxation of the pH gradient is almost unchanged, but the apparent H⁺:O ratio is decreased (Fig. 3B). Presumably, the rapid equilibration of the pH gradient formed during respiration with ammonia uptake masks proton transport and leads to an underestimation of the H⁺:O ratio. This effect may occur at neutral pH with some unidentified component of the system such as an amine or CO₂ which lowers the apparent H⁺:O ratio.

Oligomycin Titration of Proton Permeability and Respiratory Rate—The effect of oligomycin on respiration has been studied in detail and it has been concluded that oligomycin inhibits the hydrolysis of a high energy intermediate of oxidative phosphorylation which occurs when submitochondrial particles are partially deficient in coupling factors (7). The observation that oligomycin also inhibits the equilibration of a pH gradient formed during respiration suggests that oligomycin decreases the proton permeability of these particles (10). If primary proton transport is coupled to the reactions of oxidative phosphorylation by either a chemiosmotic or a chemical mechanism, then a decrease in proton permeability would be expected to decrease the rate of respiration by inhibiting cyclic coupled proton movements.

Fig. 4 shows a titration of ASU particles with oligomycin to compare the effect of this inhibitor on the respiratory rate measured with NADH as substrate as described in Figs. 1 and 2 except that 40 ng atoms of oxygen were added as oxygen-saturated ethanol so that the amount of protons transported went into a steady state before respiration ceased.

FCCP Titration of Proton Transport and Respiratory Rate—A correlation was also observed between the stimulation of respiration and the decrease in t₁/₂ for equilibration of a pH gradient when these processes were titrated with the uncoupling agent FCCP. As shown in Fig. 5, the extent of the decrease in t₁/₂ of pH decay is inversely proportional to the increase in respiratory rate. The effective proton conductance, Cₘ, is related to the t₁/₂ of pH equilibration by the equation Cₘ = (dΔpH/dH⁺) In 2 (see Reference 17), where B is differential buffering power, dΔpH/dΔH⁺. Thus, if the rate of respiration is limited by the proton conductance, it is inversely proportional to t₁/₂. At low FCCP concentration the increase in proton permeability is seen to be proportional to the FCCP concentration. A t₁/₂ of less than 2 sec is not accurately measurable because of the response time of the apparatus. Fig. 5 also shows a titration with FCCP of the maximum extent of proton influx during NADH oxidation.
and $q$ of decay of the pH gradient. The extent of proton influx is less sensitive to FCCP than the rate of decay of the gradient. Thus, the increase in respiratory rate at low FCCP concentrations maintains a high steady state of proton influx which balances the increased rate of efflux.

Measurement of Internal and External Buffering Power—In order to calculate proton permeability coefficients from the observed pH gradient decay rates, it is necessary to measure the internal and external buffering power of submitochondrial particles. This was done by rapidly changing the external pH by addition of acid or alkali and recording the "overshoot" and the decay curve that result from the relative inaccessibility of the internal buffering power (17). Carbonic anhydrase was added to avoid a small FCCP-insensitive overshoot due to the slowness of bicarbonate-CO$_2$ equilibration. These experiments showed values of 10 and 43 ng ions of H$^+$ per mg of protein × pH for the internal ($B_I$) and external ($B_E$) buffering power of ASU particles at pH 6.0, respectively, calculated according to Mitchell and Myers (17). The internal buffering power is considerably lower than the internal buffering power of rat liver mitochondria (17). This finding is not unexpected since whole mitochondria contain internal (matrix) proteins, whereas submitochondrial particles presumably contain very few soluble proteins. The measured internal buffering power decreased even lower at higher pH values. These measurements may be in error, however, from the same processes which cause the H$^+$:O ratio to decrease at pH values above 6.5. From the measured buffering powers, the differential buffering power, $B = ((1/B_E) + (1/B_I))^{-1}$ (see Reference 17) at pH 6.0 is about 10 ng ions of H$^+$ per mg of protein × pH, and a typical $q$ of equilibration of 15 sec in the presence of oligomycin gives an effective proton conductance of 0.5 μg ions of H$^+$ per g × pH × sec. This value may be compared with 0.42 μg ion of H$^+$ per g × pH × sec for rat liver mitochondria at pH 6.0 (17). An accurate estimation of the area of the submitochondrial particle membrane has not been made, although it is probably about $5 \times 10^5$ cm$^2$ per g of protein which gives a value of 1 μmho per cm$^2$ for the proton conductance per unit area.

Effect of Coupling Factors on Proton Transport—The effect of coupling factors on energy-linked functions other than ATP synthesis has been called the structural effect of coupling factors (19). These effects of coupling factors include stimulation of the reduction of NAD by succinate driven by ascorbate oxidation, the energy-linked transhydrogenase activity driven by succinate oxidation, and the induction of FCCP-stimulated respiration (10). The effect of coupling factors on the respiratory rate, on the extent of proton uptake, and on the $q$ of pH equilibration is shown in Table I. The coupling factors and ASU particles were incubated for 10 min at 25° and then dialyzed as described in Table I to remove ammonium salts. This dialysis was necessary because as little as 1 mm ammonium chloride interferes with the measurement of proton transport. After dialysis the maximum extent of proton transport driven by NADH oxidation, the $q$ of equilibration of pH gradient, and the respiratory control ratio of the rates of respiration with and without FCCP were measured with and without oligomycin present. The combination of OSCP and F$_1$ induced proton transport, followed by decay of the pH gradient with a $q$ of 7 sec. The amount of both coupling factors had been previously titrated to be about 90% optimal based on the level of respiratory control of the particles. This effect of coupling factors on proton transport observed during respiration is analogous to the stimulation of light-driven proton transport in subchloroplast particles by the chloroplast coupling factor CF$_1$ (23). A stimulation of proton transport in ASU particles by F$_1$ alone has been reported (20). The effect of F$_1$ alone presumably depends on the degree of OSCP deficiency of the particles. In our hands, a decreased proton permeability of ASU particles was only observed in the presence of both OSCP and F$_1$ unless oligomycin was present. The effect of OSCP in the presence of oligomycin is also of interest, and may indicate that there are multiple uncoupling reactions which are prevented by coupling factors and oligomycin. Protection of the particles by the coupling factors during dialysis might also explain this observation, however.

<table>
<thead>
<tr>
<th>Coupling Factors</th>
<th>FCCP respiratory control ratio</th>
<th>$H^+$ efflux</th>
<th>$q$ pH decay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minus OSCP</td>
<td>1.00</td>
<td>5.9</td>
<td>1.9</td>
</tr>
<tr>
<td>Plus OSCP</td>
<td>1.02</td>
<td>7.8</td>
<td>3.4</td>
</tr>
<tr>
<td>Minus F$_1$</td>
<td>1.04</td>
<td>7.5</td>
<td>6.2</td>
</tr>
<tr>
<td>Plus F$_1$</td>
<td>3.4</td>
<td>8.4</td>
<td>18.4</td>
</tr>
</tbody>
</table>

**Discussion**

Many proposals for the mechanism of mitochondrial ion transport have been advanced, including primary cation transport (24, 25), primary anion transport (25), primary proton transport (1, 2, 4), and electrically neutral proton-cation exchange (26). The relative merits of most of these hypotheses have been well reviewed (3, 27). There is considerable evidence that the cyclic polyphosphate valinomycin creates permeability to potassium ions in artificial membranes (28, 29) and in the mitochondrial membrane (29, 30) and that uncoupling agents of the dinitrophenol type create permeability to protons (31, 32). Thus the fact that respiration-driven K$^+$ transport requires valinomycin and is inhibited by uncoupling agents indicates that potassium ions move down their electrochemical gradient and protons are transported against their electrochemical gradient. In the experiment reported here valinomycin has been added to allow maximum proton movement by providing a permeant cation to the system. We have not measured potassium movements in these experiments but respiration-driven influx of potassium from submitochondrial particles in the presence of valinomycin has been reported (11, 19).

The $H^+:O$ ratios determined at pH 6.0 to 6.5 during brief bursts of NADH oxidation agree with $H^+:O$ ratios in rat liver mitochondria (1). At more acid or base pH, however, the maxi
Thus, the amount of protons transported to the outer face of the membrane during the burst of oscillation and the subsequent release by FCCP indicate that oligomycin or coupling factors prevent some uncoupling reaction. Previous reports that oligomycin restores proton transport to coupling-factor-deficient submitochondrial particles suggest that cyclic proton movements, where protons are transported inward and then leak out, may be the uncoupling process which is prevented by oligomycin. The concept that the respiratory rate is controlled by proton permeability (5) is supported by the correlation between proton permeability and the rate of respiration at different levels of oligomycin or the uncoupling agent FCCP. The coupling factors, F₁ plus OSCP, like oligomycin, restore proton uptake in ASU particles. This effect of coupling factors is also due to a decrease in proton permeability of the particles as shown by the slower rate of equilibration of the pH gradient when respiration ceases. The fact that the combination of coupling factors and oligomycin is more effective than either alone suggests that these particles have more than one pathway of proton (or hydroxide ion) permeability.

The effect of coupling factors on permeability to protons may reflect the sealing of a pore in the membrane when the coupling factors are attached. The fact that oligomycin also decreases proton permeability, however, suggests that there are proton-conducting groups associated with the ATPase complex which act as uncouplers when F₁ is removed, and carry protons across the membrane down their electrochemical gradient. When F₁ and OSCP are present these groups are prevented from conducting protons freely and may instead couple proton transport to the ATPase reaction, as proposed by Mitchell (4, 5).

REFERENCES


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