The Phosphorylation Potential Generated by Respiring Bovine Heart Submitochondrial Particles

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A phosphorylation potential $\Delta G_p$, where $\Delta G_p = \Delta G^\circ + RT2.303\log\left(\frac{[\text{ATP}]}{[\text{ADP}][\text{P}_i]}\right)$, of approx. 44.3 kJ mol$^{-1}$ (10.6 kcal mol$^{-1}$) was generated by submitochondrial particles that were oxidizing either NADH or succinate. Addition of adenylyl imidodiphosphate, which should suppress adenosine triphosphatase activity of any uncoupled particles, did not raise the phosphorylation potential. Raising the $\text{P}_i$ concentration slightly increased the magnitude of the value for $[\text{ATP}]/[\text{ADP}]$, but this did not fully compensate for the increased $\text{P}_i$ concentration, so that the phosphorylation potential decreased slightly as the $\text{P}_i$ concentration was raised. The phosphorylation potential developed by submitochondrial particles is lower than that generated by phosphorylating membrane vesicles from some bacteria, and is also less than that developed externally by mitochondria, but is strikingly close to the phosphorylation potential that is generated internally by mitochondria.

There is considerable evidence that mitochondria generate an external phosphorylation potential, $\Delta G_p$, where

$$\Delta G_p = \Delta G^\circ + RT2.303\log\left(\frac{[\text{ATP}]}{[\text{ADP}][\text{P}_i]}\right)$$

that is considerably higher than the phosphorylation potential in the mitochondrial matrix (see, e.g., Slater et al., 1973; Davis & Lumeng, 1975; Klingenberg, 1975). This finding indicates that the adenine nucleotide translocator is energy dependent, and probably some of the energy released by the respiratory chain is used by the translocator during ATP synthesis in intact mitochondria (Klingenberg, 1975). Submitochondrial particles, which are inside-out relative to mitochondria (Racker, 1970), have an ATP-synthesizing apparatus that is directly available to added ADP, so that the adenine nucleotide translocator should not be involved in oxidative phosphorylation, and the medium in which the particles are suspended is topologically equivalent to the matrix of intact mitochondria. It is therefore of interest to determine whether submitochondrial particles generate a phosphorylation potential that is close to either the internal or external phosphorylation potential of mitochondria. In principle the energy available from the respiratory chain should be sufficient to enable submitochondrial particles to generate the same phosphorylation potential as is found outside mitochondria. A determination of the phosphorylation potential in submitochondrial particles would also be of use in assessing the possibility that the phosphorylation potential in the mitochondrial matrix might have been underestimated if a significant fraction of the matrix ADP is usually bound (Slater et al., 1973).

To our knowledge there is only one previously reported determination of the phosphorylation potential supported by submitochondrial particles, in which Lemasters & Hackenbrock (1976) reported values of 41.0–43.9 kJ mol$^{-1}$ (9.8–10.5 kcal mol$^{-1}$) in rat liver submitochondrial particles with succinate as substrate. The lack of data on phosphorylation potentials in submitochondrial particles probably stems from the expectation that preparations of these particles will contain significant numbers of particles that are uncoupled and will therefore provide a high capacity for hydrolysing ATP, thus preventing a 'true' phosphorylation potential from being attained (cf. Eilermann & Slater, 1970). The present paper includes measurements of the phosphorylation potential made under a variety of conditions that were chosen so that the effect of any uncoupled ATPase activity could be assessed.

The reaction $\text{ADP}+\text{P}_i \rightleftharpoons \text{ATP}+\text{H}_2\text{O}$ is thought to reach equilibrium with the mitochondrial respiratory chain (Owen & Wilson, 1974; Wilson et al., 1974). A further purpose of the present paper was to see if the equilibrium $[\text{ATP}]/([\text{ADP}][\text{P}_i])$ is established by submitochondrial particles when the $\text{P}_i$ concentration is varied.

* Abbreviations: ATPase, adenosine triphosphatase; Mg–ATP particles, phosphorylating particles prepared by sonication of bovine heart mitochondria in the presence of 15 mM MgCl$_2$ and 1 mM ATP.
Materials and Methods

Bovine heart submitochondrial particles were prepared by the method of Ferguson et al. (1977) for making ATPase-inhibitor-depleted Mg-ATP particles (Type II particles in the nomenclature of Ferguson et al., 1977). Details of the procedure for determining the phosphorylation potentials are given in the legend to Table 1. The general principle of the method is that respiring submitochondrial particles are incubated for an appropriate time with ADP and P$_i$, and the final concentrations of ATP, ADP and P$_i$ are determined. For determination of P/O ratios (mol of phosphate esterified per g-atom of O consumed), submitochondrial particles were incubated in a thermostatically controlled cell at 30°C and the amount of O$_2$ consumed was measured with an oxygen electrode. ATP synthesized was determined from the incorporation of $^{32}$P$_i$ into glucose 6-phosphate, by using glucose and hexokinase as a trap for the ATP. The reaction mixtures, of total volume 4.3 ml, were basically as described in the legend to NADH or succinate was the substrate. The $^{32}$P$_i$ concentration was 10 mM (33000 c.p.m./$\mu$mol), and 0.25 mM-ADP, 10 mM-glucose and 20 units (1 unit catalyses the phosphorylation of 1.0 $\mu$mol of glucose/min at 25°C, pH 8.5) of yeast hexokinase [Sigma (London) Chemical Co., Kingston-upon-Thames, U.K.] were also added. Incorporation of $^{32}$P$_i$ into organic phosphate was determined by the method of Pullman (1967).

Adenyl imidodiphosphate, ADP, carboxyatractylsido and all enzymes (unless otherwise specified) were from Boehringer Corp. (London), Lewes, Sussex BN7 1LG, U.K., and NAD$,^+$, yeast alcohol dehydrogenase and rotenone were from Sigma. All other chemicals were of the highest quality commercially available. Protein was determined by the biuret method (Gornall et al., 1949).

Results and Discussion

Table 1 shows the results of a number of experiments in which respiring submitochondrial particles were incubated with ADP and P$_i$, and then the final concentrations of ATP, ADP and P$_i$ were measured. Since respiration of submitochondrial particles is not stimulated during phosphorylation of ADP, the point at which ATP synthesis ceases cannot be readily determined. Several lines of evidence show that the failure of the particles to phosphorylate all the added ADP was not a simple consequence of a slow rate of ATP synthesis or exhaustion of dissolved oxygen. First, similar final [ADP]/[ADP] ratios were obtained at two different concentrations of added ADP (compare Expts. 7 and 8 with 2 and 5). Secondly, when a longer incubation was made with catalase and H$_2$O$_2$ present to maintain an aerobic reaction mixture, no increase in the [ATP]/[ADP] ratio was observed. Thirdly, the typical P/O ratios for the particles (approx. 0.9 with NADH as substrate, and approx. 0.6 with succinate) were such that air-saturated buffer contained sufficient dissolved O$_2$ to allow complete phosphorylation of added ADP.

The P/O ratios of the particles used in the present work are close to the ratios obtained by others (Christiansen et al., 1969; Papa et al., 1969; Thayer & Hinkle, 1975) under comparable reaction conditions. Thayer & Hinkle (1975) showed that in submitochondrial particles the rate of phosphorylation did not vary significantly over a range of P/O ratios, so that relatively low P/O ratios can be a result of high respiration rates rather than unusually low phosphorylation rates. It was further shown (Thayer & Hinkle, 1975) that the rate of NADH oxidation was significantly stimulated (3-4-fold) by an uncoupler only under reaction conditions that gave a high P/O ratio. The relatively low P/O ratios of the particles used in the present work can also be ascribed to a high respiration rate rather than to a low rate of ATP synthesis. The rate of ATP synthesis by the particles was 310 nmol/min per mg of protein with NADH as substrate, and 160 nmol/min per mg of protein with succinate, in good agreement with the corresponding values reported by Thayer & Hinkle (1975). A further indication that our particles had a high respiration rate was that the rate of NADH oxidation catalysed by our particles was only slightly stimulated (less than 2-fold) by the uncoupler carbonyl cyanide $p$-trifluoromethoxyphenyhydrazone, just as was found by Thayer & Hinkle (1975) when their particles had a high respiration rate. In the context of the present paper it is noteworthy that the magnitude of the P/O ratio is not necessarily related to the phosphorylation potential, as a lowered stoichiometry of the phosphorylation reaction can result in the generation of a higher phosphorylation potential. If, however, the P/O ratio is lowered either as a result of respiration by non-phosphorylating particles or as a consequence of the side reactions, then the phosphorylation potential may tend to be lower than its 'true' value.

Expts. 1–3 and 4–6 in Table 1 show that oxidation of succinate and NADH generated similar phosphorylation potentials. The value of approx. 44.3 kJ·mol$^{-1}$ (10.6 kcal·mol$^{-1}$) is low in comparison with the phosphorylation potentials that are maintained externally by mitochondria (Slater et al., 1973), chloroplasts (Kraayenhof, 1969), membrane vesicles from some bacteria such as Azotobacter vinelandii (Eiellerman & Slater, 1970) and Paracoccus denitrificans (D. B. Kell, S. J. Ferguson & P. John, unpublished work), and in chromatophores from some photosynthetic bacteria (Casadio et al., 1974).
Table 1. Phosphorylation potentials generated by submitochondrial particles

Submitochondrial particles (approx. 2 mg of protein) were incubated in a reaction mixture that contained 20 mM-Tris/acetate, 5 mM-magnesium chloride, with additions from a stock potassium phosphate solution to give the required P1 concentration. The pH was 7.3, the temperature 30°C and the total volume of the reaction mixture was 4.3 ml. The reactions were started by the addition of ADP (0.230 mM in all experiments except 7 and 8, in which the ADP concentration was initially 0.150 mM). When NADH was the substrate the following additional components were added to the reaction mixture: 0.2 mg of yeast alcohol dehydrogenase, ethanol to a final concentration of 1.2% (v/v) and NAD+ (0.4 mM). When succinate was the substrate, sodium succinate (5 mM) and 5 μg of rotenone were added to the reaction mixture. In Expt. 9, 0.4 mM-adenyl imidodiphosphate, and in Expts. 10 and 11, 0.2 mM-adenyl imidodiphosphate was present throughout the reaction. In Expt. 12, bovine liver catalase (2000 units) and 20 mM-H2O2 were present throughout the reaction. The experiments were done in a thermostatically controlled cell that was fitted with a Clark-type oxygen electrode. Except in Expt. 12 the reaction was allowed to proceed until just before anaerobiosis was reached. At this point 2 ml of the reaction mixture was withdrawn and added to 0.2 ml of ice-cold 40% HClO4. In Expt. 12 the reaction was allowed to proceed for 10 min (normally anaerobiosis was reached after approx. 4 min), and then 2 ml of the reaction mixture, which was still aerobic, was added to 0.2 ml of the HClO4. The acid extracts were left on ice for 10 min, and then the precipitated protein was removed by centrifugation at 2000g. The supernatants were neutralized by addition of the predetermed amount of 0.25 m- Tris/10% (w/v) KOH, and EDTA was also added to a final concentration of 2 mM. ATP in the neutralized extracts was determined with hexokinase and glucose 6-phosphate dehydrogenase, and ADP was assayed by pyruvate kinase and lactate dehydrogenase as described by Bergmeyer (1970). P1 was measured by the method of Hurst (1964) in a Technicon Autoanalyser. In calculating ΔGp, a value for ΔG° of 30.1 kJ mol⁻¹ (7.2 kcal mol⁻¹) was used (Rosing & Slater, 1972). Each set of experimental data is an average of the results of two experiments that were carried out with the same preparation of particles.

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>Substrate</th>
<th>[ATP] (mM)</th>
<th>[ADP] (mM)</th>
<th>[P1] (mM)</th>
<th>ΔGp [kJ mol⁻¹ (kcal mol⁻¹)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NADH</td>
<td>0.157</td>
<td>0.071</td>
<td>4.4</td>
<td>45.6 (10.9)</td>
</tr>
<tr>
<td>2</td>
<td>NADH</td>
<td>0.187</td>
<td>0.053</td>
<td>9.1</td>
<td>44.7 (10.7)</td>
</tr>
<tr>
<td>3</td>
<td>NADH</td>
<td>0.197</td>
<td>0.050</td>
<td>18.4</td>
<td>45.3 (10.4)</td>
</tr>
<tr>
<td>4</td>
<td>Succinate</td>
<td>0.160</td>
<td>0.070</td>
<td>4.4</td>
<td>45.6 (10.9)</td>
</tr>
<tr>
<td>5</td>
<td>Succinate</td>
<td>0.183</td>
<td>0.060</td>
<td>9.1</td>
<td>44.7 (10.7)</td>
</tr>
<tr>
<td>6</td>
<td>Succinate</td>
<td>0.191</td>
<td>0.042</td>
<td>18.4</td>
<td>43.9 (10.5)</td>
</tr>
<tr>
<td>7</td>
<td>NADH</td>
<td>0.111</td>
<td>0.035</td>
<td>9.1</td>
<td>44.7 (10.7)</td>
</tr>
<tr>
<td>8</td>
<td>Succinate</td>
<td>0.100</td>
<td>0.029</td>
<td>9.1</td>
<td>45.1 (10.8)</td>
</tr>
<tr>
<td>9</td>
<td>NADH</td>
<td>0.177</td>
<td>0.070</td>
<td>9.1</td>
<td>44.3 (10.6)</td>
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<tr>
<td>10</td>
<td>NADH</td>
<td>0.196</td>
<td>0.057</td>
<td>9.1</td>
<td>45.1 (10.8)</td>
</tr>
<tr>
<td>11</td>
<td>Succinate</td>
<td>0.208</td>
<td>0.046</td>
<td>9.1</td>
<td>45.6 (10.9)</td>
</tr>
<tr>
<td>12</td>
<td>NADH</td>
<td>0.150</td>
<td>0.060</td>
<td>9.1</td>
<td>43.9 (10.5)</td>
</tr>
</tbody>
</table>

It may therefore be argued that the membranes of submitochondrial particles are damaged to an extent that they are unable to sustain a 'true' phosphorylation potential. In terms of the chemiosmotic hypothesis (Mitchell, 1966), the membrane may be leaky to protons or to other ions, and thus be unable to maintain a sufficiently large protonmotive force to drive the phosphorylation potential to the extent that may be expected from the free energy released during electron transport. Contrary to this view is the observation that oxidation of both NADH and succinate generated the same phosphorylation potential, although the rate of succinate oxidation was approx. 70% of the rate of NADH oxidation. This means that the rate of H⁺ translocation across the membrane with succinate would be expected to be around 50% of the rate when NADH is substrate. [This assumes that for every three protons translocated with NADH as substrate two protons are translocated when succinate is substrate (Mitchell, 1966).] Thus if submitochondrial particles are particularly leaky to protons it is surprising that similar phosphorylation potentials, and presumably similar protonmotive forces, can be maintained with both NADH and succinate despite the decrease in the rate of H⁺ translocation when succinate is oxidized instead of NADH. It is noteworthy that for mitochondria it has been shown that the protonmotive force does decline as the rate of H⁺ translocation decreases, except at the highest rates of H⁺ translocation, where the dielectric breakdown of the membrane seems to limit the potential that can be reached (Nicholls, 1974). It may be that dielectric breakdown also limits the magnitude of the protonmotive force in submitochondrial particles, but this would not be consistent with a high intrinsic proton permeability of the membrane.

It is possible that the phosphorylation potentials in Expts. 1–6 may be low because of competing ATP hydrolysis that might be catalysed by the ATPase enzyme of uncoupled non-phosphorylating particles. Two arguments counter this possibility. First, the
same potential is generated irrespective of whether NADH or succinate is used as substrate, although the rate of ATP synthesis is considerably slower with the latter substrate. Thus it would be expected that if uncoupled ATPase activity was acting to decrease the phosphorylation potential, then a lower phosphorylation potential might be expected with succinate, contrary to what is observed (Table 1). Secondly, Expts. 7-9 show that addition of adenyl imidodiphosphate did not significantly raise the phosphorylation potential. As adenylyl imidodiphosphate strongly inhibits the hydrolysis but not the synthesis of ATP by submitochondrial particles (Melnick et al., 1975; Penefsky, 1974; Pedersen, 1975), then inclusion of this inhibitor should have raised the phosphorylation potential, if uncoupled ATPase activity is normally acting to lower the phosphorylation potential. It thus appears that the magnitude of the phosphorylation potential that can be generated by submitochondrial particles is not limited by the action of uncoupled particles hydrolysing ATP; such ATP hydrolysis would lead to a steady-state phosphorylation potential governed by the relative rates of ATP synthesis and hydrolysis.

It may also be argued that the phosphorylation potential generated by submitochondrial particles is limited because some energy from the respiratory chain is partitioned into a futile operation of the adenine nucleotide translocator. This possibility can probably be ruled out, as similar phosphorylation potentials were generated by particles that had been prepared by sonicating mitochondria in the presence of 10 μm-carboxyatractyloside. In these particles any operation of the translocator should have been inhibited, as demonstrated by Lauquin et al. (1977). The finding that carboxyatractyloside did not alter the phosphorylation potential also shows that our observed phosphorylation potentials are not partly due to ATP synthesis by intact mitochondria that could contaminate the preparation of particles.

The experiments reported in the present paper indicate that the maximum phosphorylation potential that can be generated by submitochondrial particles is approx. 43.9 kJ·mol⁻¹ (10.5 kcal·mol⁻¹), and that this value is not limited by the presence of uncoupled ATPase activity or by an unduly damaged membrane. The results in Table 1 are typical; in a number of particle preparations the [ATP]/[ADP] ratio was always between 1.5 and 4.0. It is striking that the phosphorylation potential generated by submitochondrial particles is similar to the internal (matrix) phosphorylation potential that is found in mitochondria (Slater et al., 1973; Davis & Lumeng, 1975; Klingenberg, 1975). This suggests that the ATP-synthesizing apparatus in the inner mitochondrial membrane may be able to drive the ATP-synthesis reaction to a phosphorylation potential of approx. 43.9 kJ·mol⁻¹ (10.5 kcal·mol⁻¹), and that raising the phosphorylation potential beyond this value can only be achieved through operation of the adenine nucleotide translocator.

The question now arises as to whether there is an important difference between ATP synthesis by mitochondrial and bacterial membranes. Phosphorylating vesicles from bacteria can generate much higher phosphorylation potentials than submitochondrial particles. For example, vesicles from A. vinelandii (Eilermann & Slater, 1970) can maintain a phosphorylation potential of approx. 59.4 kJ·mol⁻¹ (14.2 kcal·mol⁻¹) with an [ATP]/[ADP] ratio as high as 200 (cf. Table 1). [The data of Eilermann & Slater (1970) have been recalculated by using a value for ΔG° of 30.1 kJ·mol⁻¹ (7.2 kcal·mol⁻¹).] Although the vesicles from A. vinelandii have little capacity to hydrolyse ATP (Eilermann & Slater, 1970), it seems improbable that this could account for the phosphorylation potential being higher than in submitochondrial particles, particularly in view of the arguments advanced in the present paper. Furthermore, Rhodopseudomonas capsulata chromatophores, which do have a capacity for ATP hydrolysis, can drive the phosphorylation potential to 62.7 kJ·mol⁻¹ (15 kcal·mol⁻¹) upon illumination (Casadio et al., 1974). The ability of bacterial vesicles to generate a higher phosphorylation potential than submitochondrial particles might be explicable in terms of the chemiosmotic hypothesis (Mitchell, 1966) by considering the number of H⁺ ions that are translocated across a membrane for each ATP molecule synthesized. Comparison of the protonmotive force with the phosphorylation potential in mitochondria has indicated that three H⁺ ions may be translocated for each ATP molecule synthesized (Nicholls, 1974; Wieschmann et al., 1975). Thayer & Hinkle (1973) found that in submitochondrial particles hydrolysis of, and presumably therefore synthesis of, ATP was coupled to the translocation of two H⁺ ions per ATP molecule hydrolysed. The apparent discrepancy between these two sets of observations can be accounted for if, in intact mitochondria, the extra H⁺ ion is involved in the translocation, rather than synthesis, of ATP. In bacterial vesicles it may be that higher phosphorylation potentials can be generated, compared with submitochondrial particles, because three H⁺ ions are translocated for each ATP molecule synthesized, just as has been suggested for thylakoids of chloroplasts, in which, like bacteria, no translocation of adenine nucleotides occurs (see, e.g., McCarty & Portis, 1976). Thus for a given protonmotive force a submitochondrial particle would be able to generate a phosphorylation potential approximately two-thirds that found in bacterial vesicles. This is close to the ratio found, suggesting H⁺/ATP ratios of 3 and 2 for bacterial vesicles and submitochondrial particles respectively, although
in the absence of comparative measurements of the protonmotive force this is clearly only suggestive.

If the reaction $\text{ADP} + P_i \rightleftharpoons \text{ATP} + \text{H}_2\text{O}$ reaches equilibrium with the mitochondrial respiratory chain, as suggested by Owen & Wilson (1974) and Wilson et al. (1974), then the magnitude of the phosphorylation potential should be independent of the $P_i$ concentration. Slater et al. (1973) found that with rat liver mitochondria the phosphorylation potential did depend on the $P_i$ concentration, and so the equilibrium between the respiratory chain and the ATP-synthesis reaction was not reached. It was suggested that at high $[\text{ATP}]/[\text{ADP}]$ ratios the rate-limiting step in oxidative phosphorylation may be the transport of ADP into mitochondria, so that a steady state rather than an equilibrium is reached. With submitochondrial particles it may be expected that the phosphorylation potential should not depend on the $P_i$ concentration, as no substrate-carrier systems intervene between the added substrates and the ATP-synthesizing apparatus. Expts. 1–6 (Table 1) show that raising the $P_i$ concentration did increase the ratio $[\text{ATP}]/[\text{ADP}]$, but that the increase was not sufficient to prevent a decrease in the ratio $[\text{ATP}]/([\text{ADP}][P_i])$, and hence in the phosphorylation potential. These data therefore do not provide good supporting evidence for the idea that the ATP-synthesis reaction reaches equilibrium with the respiratory chain. Indeed the data presented in Table 1 leave open the possibility that the magnitude of the phosphorylation potential could be limited by the activity of the mitochondrial ATPase, which might be controlled by the $[\text{ATP}]/[\text{ADP}]$ ratio. In this respect it is noteworthy that van de Stadt et al. (1973) suggested that the activity of the ATPase could be controlled via an interplay between the $[\text{ATP}]/[\text{ADP}]$ ratio and the energized state of the membrane. Such a mechanism could also provide an alternative explanation for the finding that submitochondrial particles support a lower phosphorylation potential than bacterial vesicles. It might be that the mechanism of control of the ATPase is different in bacteria.

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Vol. 168