# Energetics of ATP-Driven Reverse Electron Transfer from Cytochrome *c* to Fumarate and from Succinate to NAD in Submitochondrial Particles†

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ABSTRACT: The maximum Gibbs free energies of reverse electron transfer from succinate to $NAD^+$ and from cytochrome *c* to fumarate driven by ATP hydrolysis in submitochondrial particles from beef heart were measured as a function of the Gibbs free energy of ATP hydrolysis. The ratio of the energies $\Delta G'_{redox}/\Delta G'_{ATP}$ was 1.40 from succinate to $NAD^+$ and 0.89 from cytochrome *c* to succinate. The ratio, equivalent to a thermodynamic $P/2e^-$ ratio, was dependent on whether the electrochemical proton gradient was primarily a membrane potential or a pH gradient for the cytochrome *c* to fumarate reaction. The results are consistent with $H^+/ATP = 3$ for $F_1$ ATPase, $H^+/2e^- = 4$ for NADH-CoQ reductase, and $H^+(matrix)/2e^- = 2$ for succinate-cytochrome *c* reductase.

It has long been recognized that oxidative phosphorylation is readily reversible at the first two coupling regions (Chance & Hollunger, 1961; Klingenberg & Schollmeyer, 1961). Energetic analyses of oxidative phosphorylation have been made from NADH to cytochrome *c* in mitochondria (Forman & Wilson, 1982; Erecinska et al., 1974) and from NADH to fumarate in submitochondrial particles (Rottenberg & Gutman, 1977; DeJong & Westerhoff, 1982). The former studies indicated a $P/2e^-$ ratio of 2 from NADH to cytochrome *c* and the latter a $P/2e^-$ ratio of 1.3 from NADH to fumarate. Other studies (Rottenberg, 1979; Stucki, 1980; Lemasters & Billica, 1981) have included the third coupling region which is not readily reversible (Bienfait, 1975) and thus less amenable to analysis. There is agreement that electron transfer from succinate to cytochrome *c* causes the disappearance of two protons inside mitochondria, the efflux of two positive charges across the membrane measured as $K^+$ uptake in the presence of valinomycin, and the appearance of four protons outside of mitochondria, the extra two protons outside being the net acid formation from the overall reaction (Mitchell & Moyle, 1967; Leung & Hinkle, 1975; Alexandre & Lehninger, 1979). The energetics of oxidative phosphorylation in this region are thus of particular interest because they should allow calculation of the $H^+/ATP$ ratio of ATP synthesis in addition to the P/O ratio. Well-coupled inverted submitochondrial particles are appropriate for this analysis because the succinate/fumarate couple and ATP interact with enzymes on the outside surface of the vesicles. This allows external concentrations to be used unambiguously in the calculations and avoids the coupling of ATP/ADP exchange to the membrane potential ($\Delta\psi$) and the transport of $P_i$ and succinate to the pH gradient ($\Delta$pH) which occurs in mitochondria [e.g., see Klingenberg (1972)]. The fact that cytochromes $c + c_1$ are inside submitochondrial particles also introduces the interesting prediction that the equilibrium position of reverse electron transfer should depend on whether the electrochemical proton gradient is primarily a membrane potential or a pH gradient.

Here we report studies of the equilibria of ATP-driven reverse electron transfer at the first and second coupling regions of the respiratory chain in submitochondrial particles which are relevant to the determination of the stoichiometries of proton transport and ATP synthesis.

## Materials and Methods

Electron transport particles prepared by sonication of heavy-layer bovine heart mitochondria ($ETP_H$)[1] ($Mg^{2+}$, $Mn^{2+}$) were prepared from beef heart mitochondria as described previously and stored at −70 °C (Hansen & Smith, 1964). ATP, ADP, enzymes, buffers, and inhibitors were from Sigma. The uncoupler SF6847 was a gift from Y. Nishizawa, Sumitomo Chemical Co., Ltd., Osaka, Japan.

*Reverse Electron Transfer at the Second Site.* The redox level of cytochromes $c + c_1$ was measured at 550–540 nm with an Aminco dual-wavelength spectrophotometer. Some experiments were also done at 552–540 nm, the peak of cytochrome $c + c_1$ adsorption in $ETP_H$, with very similar results. A 3-mL cuvette was used in a thermostated cell holder at 37 °C. Experiments at room temperature were found to have less effective reverse electron transfer driven by the same $\Delta G'_{ATP}$, but the effect of a given $\Delta G'_{ATP}$ was the same at 25 and 37 °C. This probably reflects the large temperature coefficient of $F_1$–$F_0$ ATPase.

For second-site $\Delta G'_{ATP}$ titrations, $ETP_H$ (0.5 mg/mL) were added to a medium of 250 mM sucrose, 50 mM K-Mops, pH 7.1, 5 mM $MgSO_4$, and 1 mg/mL bovine serum albumin. Further additions of KCN (5 mM), potassium succinate (1–5 mM), and potassium fumarate (5–25 mM) were made with a "plumper" (Calbiochem). At this point, for all ratios of succinate/fumarate used, cytochromes $c + c_1$ were still 100% reduced, and the pH was 7.1. ATP, $MgSO_4$, ADP, and $P_i$ (pH 7.1) were then added at levels of from 1 to 10 mM, and cytochromes $c + c_1$ became more oxidized within 15 s. $MgSO_4$ was added with the different mixtures of ATP, ADP, and $P_i$ used so that the free $Mg^{2+}$ concentration was always 5 mM. The amount of $MgSO_4$ was calculated by using a computer program (Kohlbrenner & Cross, 1979) and association constants from Storer & Cornish-Bowden (1976) and Rosing & Slater (1972). A sample (0.5 mL) of the solution was withdrawn after 2 min, fixed with 0.25 mL of 21% perchloric acid, added to 0.25 mL of 0.8 M MES/0.8 M Mops, titrated to a pH value between 6 and 7 with 6 N KOH, and stored on ice for determination of ATP (Lamprecht & Trautchold, 1974), ADP, and AMP (Jaworek et al., 1974) concentrations the

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[1] Abbreviations: $ETP_H$, electron-transport particles prepared by sonication of heavy-layer bovine heart mitochondria; SF6847, (3,5-di-*tert*-butyl-4-hydroxybenzylidine)malononitrile; MES, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid.

0006-2960/84/0423-3341$01.50/0 

same day. At values of $\Delta G_{ATP}$ around −45 kJ/mol, the value remained constant over a period of 3 min, whereas values of −50 kJ/mol decreased to −48 kJ/mol and values of −35 kJ/mol increased to −36 kJ/mol due to ATP formation by adenylate kinase. After the sample was taken, 1 μM SF6847 was added to uncouple the submitochondrial particles, and cytochromes $c + c_1$ became 100% reduced. $\Delta G'_{ATP}$ was calculated from the equation

$$\Delta G'_{ATP} = \Delta G^{\circ\prime}{}_{ATP} + RT \ln \frac{[ADP][P_i]}{[ATP]}$$

where $\Delta G^{\circ\prime}{}_{ATP}$ was obtained by interpolation from values reported by Rosing & Slater (1972) and varied from −28.7 to −29.0 kJ/mol ($T$ = 37 °C) depending on the ionic strength. An independent determination of $\Delta G^{\circ\prime}{}_{ATP}$ (Guynn & Veech, 1973) found values about 1.5 kJ/mol more negative than those we are using. Using this higher $\Delta G^{\circ\prime}{}_{ATP}$ would simply shift the $x$ axis in Figures 2 and 4 to the left. The values $\Delta G'_{\text{succ-cyt } c+c_1}$ were calculated from the equation

$$\Delta G'_{\text{succ-cyt } c+c_1} = 2F[E'_{m(succ)} - E'_{m(\text{cyt } c+c_1)}] + RT \ln \frac{[\text{fumarate}]}{[\text{succinate}]} - 2RT \ln \frac{[\text{cyt } c + c_1(ox)]}{[\text{cyt } c + c_1(red)]}$$

where $F$ is the Faraday constant (96490 C/mol), $R$ is the ideal gas constant (8314 J mol$^{-1}$ deg$^{-1}$), $T$ is the absolute temperature, $E'_{m(\text{cyt } c+c_1)}$ = 227 mV (Dutton et al., 1970), and $E'_{m(succ)}$ = 17 mV at pH 7.1 (Clark, 1960).

*Reverse Electron Transfer at the First Site.* The experiment and medium were the same as for reversal at the second site, except that 5 μM $NAD^+$ was added and NADH formation was measured by the fluorescence in an Eppendorf fluorometer at 25 or 37 °C. After the steady-state reduction level of $NAD^+$ was reached, 1 μM SF6847 was added which caused reoxidation of NADH to the oxidized base line. The fully reduced signal from NADH was then measured by addition of 0.2 mL of ethanol and 45 μg of alcohol dehydrogenase, taking into account the effect of dilution by the ethanol. The values of $\Delta G'_{NADH\text{-}fum}$ were calculated from

$$\Delta G_{NADH\text{-}fum} = 2F[E'_{m(NADH)} - E'_{m(succ)}] + RT \ln \frac{[\text{succinate}]}{[\text{fumarate}]} + RT \ln \frac{[NAD^+]}{[NADH]}$$

where $E'_{m(NADH)}$ = −320 mV at pH 7.1 (Clark, 1960).

## Results

It is expected that in submitochondrial particles only the internal cytochromes $c + c_1$ will be correctly oriented to be coupled to the electrochemical proton gradient, and thus be oxidized when $\Delta\tilde{\mu}_{H^+}$ is formed by the hydrolysis of ATP by $F_1$. The submitochondrial particles used in this study oxidize added NADH with a respiratory control ratio of 5 to 10 [respiration in the presence of uncoupler divided by respiration without uncoupler in a sucrose/low-salt medium with polylysine (Berry & Hinkle, 1983)], have directly measured P/O ratios of 2.2 with NADH as substrate (Thayer & Hinkle, 1975), and by electron microscopy are found to be predominantly covered with $F_1$ knobs, indicating that they are functionally inverted with respect to mitochondria. However, several components of the respiration chain are "scrambled" by the sonication procedure (Eytan et al., 1975). To quantitate the fraction of cytochromes $c + c_1$ which is external, we have measured the reduction of these cytochromes by added ascorbate, compared with ascorbate plus phenazine methosulfate, a permeant redox mediator. The results are shown in Figure

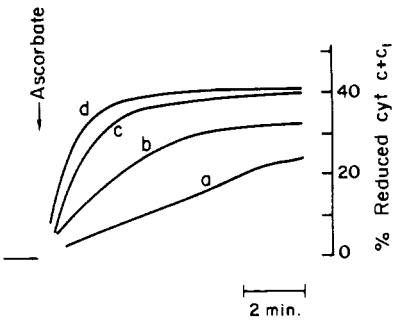


FIGURE 1: Determination of external cytochromes $c + c_1$. Reduction of external cytochromes $c + c_1$ was measured at 552–540 nm. $ETP_H$ were suspended at 1 mg/mL in 250 mM sucrose, 50 mM K-Mops (pH 7.2), and 1 mM $MgSO_4$. KCN (0.5 mM) was added, causing no significant change in the trace (not shown), followed by ascorbate at (a) 0.17, (b) 0.5, (c) 1, or (d) (most rapid) 2 mM. Reduction of total cytochromes $c + c_1$ was caused by subsequent addition of 1 μM phenazine methosulfate (not shown) to give 100% reduction.

1. The addition of ascorbate after cyanide, which inhibits cytochrome $c$ oxidase, caused reduction of 40% of cytochromes $c + c_1$ measured at 552–540 nm. Different concentrations of ascorbate caused different rates of cytochrome $c + c_1$ reduction as expected, but the maximum extent was always about 40% of that reduced by ascorbate + phenazine methosulfate or by dithionite. We assume that the external ascorbate-reducible cytochromes $c + c_1$ are not coupled to the proton gradient and are thus completely reduced by succinate under the conditions of our experiments. External cytochromes $c + c_1$ would be coupled if some vesicles in the preparation had mitochondrial sidedness, but in such vesicles, ATP would have to enter via the ATP transporter to be hydrolyzed by $F_1$ and the effects of ATP which we observe are completely resistant to 1 μM atractyloside. Our assumption is also supported by an analysis of the data (not shown) where the fraction of cytochromes $c + c_1$ not coupled was varied in the calculation until the ratio of $\Delta G'_{ATP}$ to $\Delta G'_{\text{suc-cyt } c+c_1}$ was constant through the titration (see below). The values obtained for the fraction not coupled were within 10% of that reducible by external ascorbate.

To measure the equilibrium position of reverse electron transfer at the second coupling region, mixtures of ATP, ADP, and $P_i$ were added to submitochondrial particles equilibrated with succinate/fumarate mixtures and cyanide, and the redox level of cytochromes $c + c_1$ was measured by dual-wavelength spectrophotometry. The difference between the redox potentials of internal cytochromes $c + c_1$ and succinate was then calculated as described under Materials and Methods and plotted (Figure 2) as a function of $\Delta G'_{ATP}$, the Gibbs free energy of ATP under the conditions of the experiment. The lines on Figure 2 are the expected results if $\Delta\tilde{\mu}_{H^+}$ was entirely $\Delta pH$ or $\Delta\psi$ and the $H^+/ATP$ ratio of the $F_1$–$F_0$ ATPase was 2 (dashed lines) or 3 (solid lines). The different predicted values for the equilibrium of reverse electron transfer at the second site when $\Delta\tilde{\mu}_{H^+}$ is $\Delta\psi$ or $\Delta pH$ only occur in submitochondrial particles where functional cytochromes $c + c_1$ are on the inside. The reaction is

$$\text{succinate}_{out} + 2\text{cyt } c_{in}{}^{ox} + 2H^+{}_{out} \rightleftharpoons \text{fumarate}_{out} + 2\text{cyt } c_{in}{}^{red} + 4H^+{}_{in}$$

For each electron pair transferred from external succinate to internal cytochromes $c + c_1$, four protons appear inside and are influenced by the internal pH. However, the inward flux of four protons and two electrons gives a net influx of only two positive charges which are influenced by $\Delta\psi$. Thus, the driving force on reverse electron transfer at the second site is $F(\Delta\psi) - 2RT \ln 10(\Delta pH)$. The system could also be analyzed for-

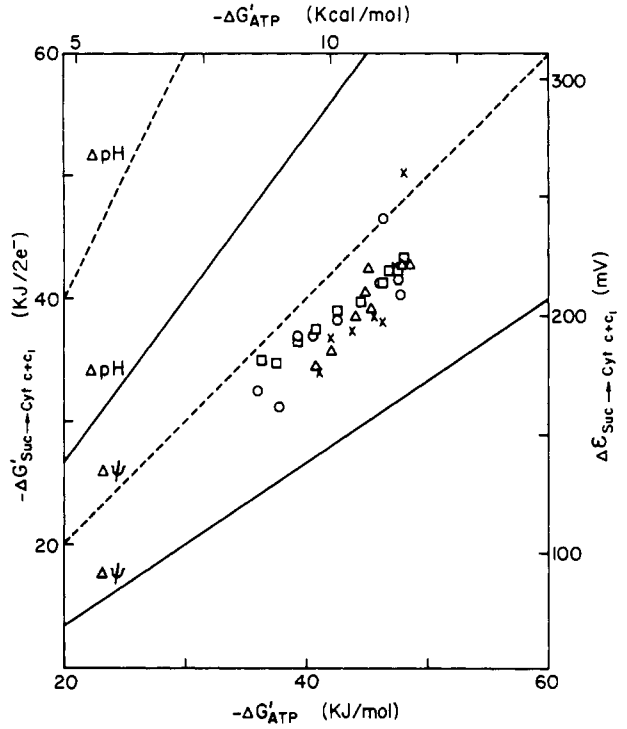


FIGURE 2: Energetics of reverse electron transfer at site 2. The difference in redox potential between succinate and internal cytochromes $c + c_1$ (calculated by using the external pH) is plotted as a function of $-\Delta G'_{ATP}$ of added ATP, ADP, and $P_i$. The data points indicate [fumarate]/[succinate] ratios of 1 (○), 3 (□), 10 (△), and 25 (×). The lines are the expected equilibrium values of reverse electron transfer if $H^+/ATP = 3$ (solid lines) or $H^+/ATP = 2$ (dashed lines) and if $H^+/2e^- = 2$ ($\Delta\psi$) or $H^+/2e^- = 4$ ($\Delta$pH).

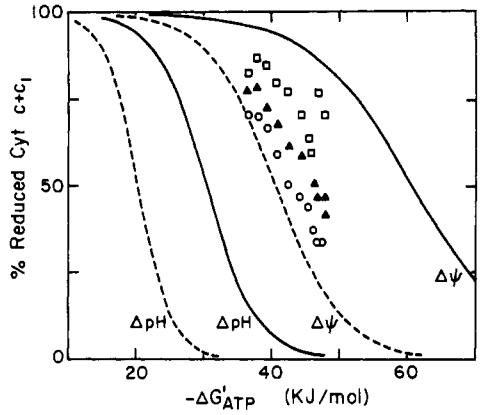


FIGURE 3: Differential effect of $\Delta$pH and $\Delta\psi$ on reverse electron transfer at site 2. Experimental conditions are given under Materials and Methods. The [fumarate]/[succinate] ratio was 3. The points are in the presence of 5 mM $(NH_4)_2SO_4$ (○) or 1 mM $NaClO_4$ (□) or no added permeant ion or base (▲). The lines are expected equilibrium values if $H^+/ATP = 3$ (solid lines) or 2 (dashed lines) and if $H^+/2e^- = 2$ ($\Delta\psi$) or 4 ($\Delta$pH).

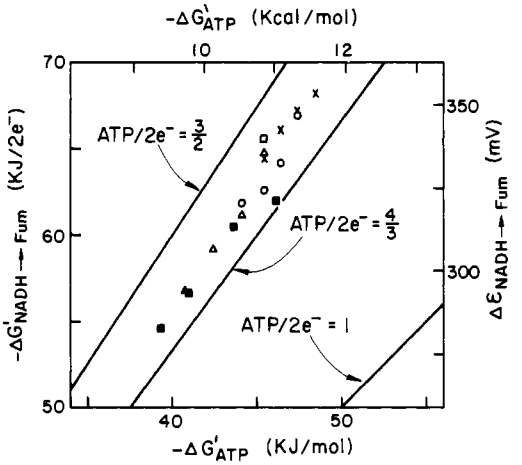


FIGURE 4: Energetics of reverse electron transfer at site 1. Experimental conditions are given under Materials and Methods. The [fumarate]/[succinate] ratio was 0.1 (□, ■), 0.4 (△), 1 (○), or 5 (×). All measurements were at 25 °C except points marked (■) which were at 37 °C. The lines are expected equilibrium values for the $ATP/2e^-$ ratios indicated.

mally in other ways, but the result would be the same. The data points at all values of $\Delta G'_{ATP}$ and [fumarate]/[succinate] lie between the predicted lines for $\Delta\psi$ and $\Delta$pH at $H^+/ATP = 3$. In other words, the average $ATP/2e^-$ ratio calculated from the energetics was $0.89 \pm 0.05$, and the predicted values of $ATP/2e^-$ when $H^+/ATP = 3$ are 1.33 if $\Delta\tilde{\mu}_{H^+}$ is entirely $\Delta$pH and 0.66 if $\Delta\tilde{\mu}_{H^+}$ is entirely $\Delta\psi$, whereas for $H^+/ATP = 2$, $ATP/2e^- = 2$ or 1 when $\Delta\tilde{\mu}_{H^+}$ is entirely $\Delta$pH or $\Delta\psi$, respectively. The measured value is consistent with $H^+/ATP = 3$ if $\Delta\tilde{\mu}_{H^+}$ is $66 \pm 8\%$ $\Delta\psi$, which is the same as the previously measured value of 65% $\Delta\psi$ with no added permeant anions or bases [see Figure 8 of Berry & Hinkle (1983)].

To demonstrate that the extent of reverse electron transfer from cytochromes $c + c_1$ to fumarate is dependent on the fraction of $\Delta\tilde{\mu}_{H^+}$ that is $\Delta\psi$, we have varied the composition of $\Delta\tilde{\mu}_{H^+}$ by addition of ammonium ions to decrease $\Delta$pH and increase $\Delta\psi$ and perchlorate ions to increase $\Delta$pH and decrease $\Delta\psi$. Figure 3 shows the effects of 10 mM $NH_4^+$ and 1 mM $ClO_4^-$ on the extent of cytochrome $c + c_1$ oxidation as a function of $\Delta G'_{ATP}$. The theoretical lines in Figure 3 correspond to those in Figure 2. Ammonia is a permeant base and was shown to increase $\Delta\psi$ from 65% to 70% of $\Delta\tilde{\mu}_{H^+}$ without changing $\Delta\tilde{\mu}_{H^+}$ (Berry & Hinkle, 1983). The permeant anion $ClO_4^-$ at 1 mM was shown to decrease $\Delta\psi$ to 50% of $\Delta\tilde{\mu}_{H^+}$ without changing $\Delta\tilde{\mu}_{H^+}$ (Berry, 1981). When the data of Figure 3 are analyzed as in Figure 2, the increased oxidation of cytochromes $c + c_1$ in the presence of $ClO_4^-$ and the opposite effect of $NH_4^+$ correspond to 63% $\Delta\psi$ with $ClO_4^-$, 73% $\Delta\psi$ without $ClO_4^-$ or $NH_4^+$, and 85% $\Delta\psi$ with $NH_4^+$, in reasonable agreement with the measured values.

Rottenberg & Gutman (1977) measured the energetics of reverse electron transfer from succinate to $NAD^+$ and obtained results compatible with transport of 4 $H^+/2e^-$ by the respiratory chain and 3 $H^+/ATP$ by $F_1$–$F_0$ ATPase, giving a theoretical $P/2e^-$ ratio of 1.33. We have made similar measurements at the first site, but instead of measuring rates of $NAD^+$ reduction at various $NAD^+/NADH$ ratios, we have simply added low concentrations of $NAD^+$ and measured the maximum steady-state level of NADH as a function of $\Delta G'_{ATP}$. The results are shown in Figure 4. In this case, both the $H^+/2e^-$ ratio of proton transport at the first site and the $H^+/ATP$ ratio of $F_1$–$F_0$ ATPase are in question, so we have drawn equilibrium lines for 2 or 3 $H^+/ATP$ and 2, 3, or 4 $H^+/2e^-$, giving possible $ATP/2e^-$ ratios of 1, $^4/_3$, or $^3/_2$. As at the second site, the overall reaction from NADH + $H^+$ to fumarate involves a net pH change. Since both NADH and fumarate react at the external surface of the membrane, this net proton change occurs in the external medium and is accounted for by using the external pH when calculating the midpoint potentials of NADH and succinate. The number of protons leaving the inner aqueous phase and the number of charges crossing the membrane during $NAD^+$ reduction are equal, and so only one predicted equilibrium line is necessary in Figure 4 for each possible stoichiometry, irrespective of the composition of $\Delta\tilde{\mu}_{H^+}$. The data points in Figure 4 are between the lines for $ATP/2e^- = {}^3/_2$ and $^4/_3$. If we use the

value of $\Delta G^{\circ\prime}_{ATP}$ reported by Guynn & Veech (1973), the data lie directly on the $ATP/2e^- = {}^4/_3$ line (see Materials and Methods). The measurement confirms the results of Rottenberg & Gutman (1977) and is consistent with four protons transported per electron pair at the first site if the $H^+/ATP$ ratio of $F_1$–$F_0$ ATPase is 3.

## Discussion

We have used the segment of the respiratory chain from succinate to cytochrome *c* as a standard against which the $F_1$–$F_0$ $H^+$ ATPase can be measured, because it is the only segment of the chain about which there is agreement on proton transport stoichiometry. The results are consistent with an $H^+/ATP$ ratio of 3. Such a calculation is justified if the overall system of proton transport by the ATPase and reversal of electron transfer by the proton gradient is at or close to equilibrium. Since there is a low proton permeability of the membrane, however, the system cannot come to a true equilibrium state. One way to estimate how close the system comes to equilibrium is to measure $\Delta G'_{\text{succ-cyt } c+c_1}$ and $\Delta G'_{ATP}$ during oxidative phosphorylation in the forward direction (during respiration) and compare the results with those of reverse electron transfer. In such measurements at equal fumarate and succinate concentrations, the total cytochromes $c + c_1$ were 15% reduced with ATP addition and 20% reduced without ATP addition. If it is assumed that the external cytochromes $c + c_1$ are completely oxidized in the steady state, these results indicate that $\Delta G'_{\text{succ-cyt } c+c_1} = 49$ kJ/$2e^-$, somewhat higher than that obtained during reverse electron transfer (Figure 2) and not very sensitive to $\Delta G'_{ATP}$. It does not seem reasonable to assume any value for the reduction level of external cytochromes $c + c_1$ in the steady state, however, and so we have not pursued such measurements. The degree of equilibration of reverse electron transfer and the ATPase can be estimated from measurements of $\Delta\tilde{\mu}_{H^+}$. Berry & Hinkle (1983) measured the equilibration between the proton gradient and ATPase in $ETP_H$ and found that the $\Delta\tilde{\mu}_{H^+}$ formed by ATP hydrolysis in the presence of ADP and $P_i$ mixtures alone was 93% of that formed at the same value of $\Delta G'_{ATP}$ but during NADH oxidation. Thus, the correction to true equilibrium might be approximated by shifting the data 3 kJ/mol less negative on the $\Delta G'_{ATP}$ axis of Figures 2 and 4.

An imbalance between $\Delta\psi$ and $\Delta$pH occurs during reverse electron transport at the second site when the chain can turn over only once. It would not occur during normal oxidative phosphorylation where the reactions of the respiratory chain and the $F_1$–$F_0$ ATPase must be balanced electrically and chemically with respect to the internal aqueous phase. In mitochondria where two protons disappear from the inside and two charges cross the membrane, there is no imbalance, and the driving force on reverse electron transfer is simply $\Delta\tilde{\mu}_{H^+}$. Four protons do appear in the external phase, but the extra two protons are accounted for when the redox potential of succinate is calculated by using the external pH. Each of the three coupling sites has scalar proton formation or absorption when assayed alone in addition to proton transport. If this net proton change occurs in the external phase, then it is included in the energetic calculation when the pH of the external phase is used to calculate potentials of the substrates and products. If the net proton change occurs in the inner phase, as in submitochondrial particles, then an extra term has to be added.

Another question is whether the net proton changes contribute to the formation of $\Delta$pH. If they occur in the external medium, then they do not contribute to $\Delta$pH because that phase is well buffered and does not change pH. If the $H^+/ATP$ ratio of the $F_1$–$F_0$ ATPase is 3 and one proton is coupled to the transport of ADP and $P_i$ into mitochondria, then the theoretical $P/2e^-$ ratio at the second site would be 0.5 in mitochondria, independent of the composition of $\Delta\tilde{\mu}_{H^+}$. There is no second-site assay for oxidative phosphorylation in submitochondrial particles because a suitable dye to oxidize internal cytochrome *c* has not been found. To work, such a dye should be a hydrogen carrier which would absorb the scalar protons formed inside, giving an expected $P/2e^-$ ratio of 0.66 independent of the composition of $\Delta\tilde{\mu}_{H^+}$.

Our confirmation of the results of Rottenberg & Gutman (1977) on the equilibrium of reverse electron transfer at the first site shows that the number of protons transported per electron pair is greater than that transported by the ATPase and is probably 4. In a similar study that included measurement of $\Delta\tilde{\mu}_{H^+}$, DeJonge & Westerhoff (1982) concluded that the stoichiometry at site 1 was 3 $H^+/2e^-$. They did not correct the measurements of $\Delta\tilde{\mu}_{H^+}$ for probe binding which is significant in submitochondrial particles (Berry & Hinkle, 1983), and so overestimated $\Delta\tilde{\mu}_{H^+}$ which would cause an underestimation of the $H^+/2e^-$ ratio.

Forman & Wilson (1982) measured the energetics of oxidative phosphorylation in mitochondria from 3-hydroxybutyrate to cytochrome *c* during respiration and during ATP-driven reverse electron transfer. They concluded that the ratio $\Delta G'_{\text{BOH-cyt } c}/\Delta G'_{ATP}$ was 2.0, whereas our results imply that in mitochondria the ratio should be 1.5 ($H^+/2e^- = 6$, $H^+/ATP = 4$). Their calculation is not valid, however, because they used the internal pH to calculate the $E'_m$ of 3-hydroxybutyrate and did not include a term of the net efflux of protons and electrons which occurs at site 2 as discussed above. Although it is not necessary to specify the mechanism (e.g., chemiosmotic) of a coupling site for a thermodynamic analysis, the products and reactants must all be specified. Thus, either the external pH should be used when calculating the $E'_m$ of 3-hydroxybutyrate or an additional term for the transport of two protons out of the matrix should be included, which amounts to the same thing. When this is done, the measured $ATP/2e^-$ ratio is significantly below 2, and other uncertainties such as $\Delta G^{\circ\prime}_{ATP}$ could make it as low as 1.5.

An incorrect analysis of the second site was also made by Pietrobon et al. (1981), who used an $H^+/2e^-$ ratio of 4 for the effect of proton transport from succinate to cytochrome *c* on the formation of $\Delta\tilde{\mu}_{H^+}$ in mitochondria. As discussed above, the charge transport and proton flux from inside mitochondria are only 2 $H^+/2e^-$, although 4 $H^+/2e^-$ are formed outside, so that the appropriate stoichiometry is 2 $H^+/2e^-$. Thus, although there is agreement about the stoichiometry of proton transport at the second site, the scalar protons have complicated the situation and made the analysis of coupling phenomena nontrivial and more interesting.

**Registry No.** ATP, 56-65-5; NAD, 53-84-9; cytochrome *c*, 9007-43-6; cytochrome $c_1$, 9035-42-1; succinic acid, 110-15-6; fumaric acid, 110-17-8.

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