Thermodynamics of Oxidative Phosphorylation in Bovine Heart Submitochondrial Particles*

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The rates of both forward and reverse electron transfer in phosphorylating submitochondrial particles from bovine heart can be controlled by the thermodynamic phosphorylation potential ($\Delta G_p$) of the adenine nucleotide system. $\Delta G_p$ is the Gibbs free energy of ATP synthesis and is defined by the relationship $\Delta G_p = -\Delta G^\circ_p + RT\ln([ATP]/[ADP][Pi])$ where $\Delta G^\circ_p$ is the standard free energy of ATP hydrolysis. Studies of the effects of $\Delta G_p$ on NADH respiration and the reduction of NAD$^+$ by succinate show that increasing values of $\Delta G_p$ cause an inhibition of forward electron transfer and a stimulation of reverse electron transfer. Between $\Delta G_p$ values of 7.6 and 13.0 kcal/mol the rate of NADH respiration decreased 3-fold and the rate of NAD$^+$ reduction by succinate increased 3-fold. Indirect phosphorylation potential titration experiments as well as direct chemical measurements indicate that steady state levels of ATP, ADP, and Pi are established during NADH respiration which correspond to a $\Delta G_p^\circ$ equal to 10.7 to 11.4 kcal/mol.

The reversible coupling between electron transfer, ATP synthesis, and the electrochemical proton gradient has been extensively analyzed in intact mitochondria (1-9). Under some experimental conditions, oxidative phosphorylation is close enough to thermodynamic equilibrium that the rates of electron flow and ATP synthesis are controlled by the overall Gibbs free energy of the phosphorylation system rather than by the concentration of a single substrate such as ADP (6, 8-12). The Gibbs free energy of ATP hydrolysis, against which phosphorylation of ADP occurs, is $\sim 16$ kcal/mol in the extramitochondrial medium (15-17) since $\Delta G_p$ values in the range of 11 to 12 kcal/mol have been observed for the intramitochondrial space (6, 8, 14). In this paper we report studies of the effect of the thermodynamic phosphorylation potential, or Gibbs free energy of ATP synthesis, on the rates of forward and reverse electron transfer in phosphorylating submitochondrial particles. These provide a simplified experimental system compared to intact mitochondria because the membrane orientation is inverted, placing the ATPase in direct contact with the external medium.

EXPERIMENTAL PROCEDURES

Preparations - ETP$_0$(Mg$^{2+}$, Mn$^{2+}$)$^1$ (18) submitochondrial particles were prepared by sonicatation of heavy layer bovine heart mitochondria (19) as previously described (20) and resuspended in 0.25 M sucrose.

Analytical Procedures - Respiratory rates were measured with a Clark oxygen electrode in a closed chamber. A standard fluorimetric procedure was used to measure the energy-linked reduction of NAD$^+$ by succinate (21). ATP and ADP were measured enzymatically (22) in aliquots of 0.5 ml of reaction mixtures quenched by 2 ml of phenol:chloroform:isoamyl alcohol (58:24:1) (6).

RESULTS

Control of Forward Electron Transfer by $\Delta G_p$ - The influence of $\Delta G_p$ on respiration was studied by recording the respiratory rates of submitochondrial particles before and after the addition of mixtures of ATP and ADP producing different $\Delta G_p$ values in the medium (Fig. 1). In order to observe the maximum effect on respiration, poly(L-lysine) was included in the medium to inhibit the oxidation of exogenous cytochrome c (23). However, the inhibition of oxygen uptake by poly(L-lysine) was less than 10% with the ETP$_0$ used in these experiments, suggesting that this submitochondrial particle preparation consists of nearly homogeneous inverted membranes (23). The altered respiratory rates remained constant for at least 1 min following the adenine membrane. The transport of ATP, ADP, and Pi in and out of the matrix space may cause the high $\Delta G_p$ of the external medium (15-17) since $\Delta G_p$ values in the range of 11 to 12 kcal/mol have been observed for the intramitochondrial space (6, 8, 14).

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§ The abbreviations used are: ETP$_0$, electron transport particles prepared from heavy layer bovine heart mitochondria; Hepes, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid.
nucleotide addition. Analysis of the respiratory rates as a function of $\Delta G_p$ showed that similar rates were obtained by either varying the ATP/ADP ratio at constant $P_i$ concentration, or by varying the $P_i$ concentration at a constant ATP/ADP ratio (Fig. 2). However, at $P_i$ concentrations greater than 10 mM additional stimulation of respiration occurred due to a salt effect (24). The $\Delta G_p$ values were calculated from the added ATP, ADP, and $P_i$ concentrations and the approximate value of $\Delta G_{pK}$, which was $-7.3 \pm 0.1$ kcal/mol for the conditions of these experiments (25). Low $\Delta G_p$ values caused a stimulation while high $\Delta G_p$ values produced an inhibition relative to the respiratory rate observed before the addition of adenine nucleotides. The rate of respiration changed approximately 3-fold between $\Delta G_p$ values of 7.6 and 13.0 kcal/mol. Addition of an uncoupler stimulated respiration 5-fold relative to the respiratory rate observed before the addition of adenine nucleotides. According to the observed relationship, the $\Delta G_p$, which caused no change in the rate of respiration, the "null point," corresponded to 10.6 kcal/mol (Fig. 2). For five independent experiments with different ETP$_c$, preparations, the null point $\Delta G_p$ occurred at 10.7 $\pm$ 0.3 kcal/mol.

Control of Reverse Electron Transfer by $\Delta G_p$—The energy-linked reduction of NAD$^+$ by succinate was examined to determine the effect of $\Delta G_p$ on reverse electron transfer. The rate of NAD$^+$ reduction was recorded following the addition of various ATP plus ADP mixtures. An approximately linear dependence of the rate of this reaction on $\Delta G_p$ was observed within the range of 8.5 and 13.0 kcal/mol (Fig. 3). In contrast to respiration, reverse electron transfer proceeded faster at high $\Delta G_p$ values and slower at low $\Delta G_p$ values. The rate of NAD$^+$ reduction increased 3-fold between $\Delta G_p$ values of 8.5 and 13.0 kcal/mol. For comparison, the rate of NAD$^+$ reduction driven by respiration (phenazine methosulfate plus ascorbate) was determined in the presence of an optimum level of the oxidation-reduction mediator phenazine methosulfate. This respiration-driven rate was equivalent to that produced by a $\Delta G_p$ of approximately 10.3 kcal/mol based on the relationship shown in Fig. 3.

Time Course of $\Delta G_p$ during NADH Respiration—In order to determine directly the value of $\Delta G_p$ normally maintained by respiration, the time-dependent change of $\Delta G_p$ from initial states having either ATP or ADP alone was analyzed during NADH respiration (Fig. 4). Vigorous aeration was necessary in order to prevent anaerobiosis during the experiment. For calculation of the $\Delta G_p$, values, the change in $P_i$ concentration was determined from the changes in ADP and ATP levels, ignoring a small formation of AMP by adenylate kinase. Starting with only ATP added to the medium, the $\Delta G_p$ declined rapidly from an initial value of 12.6 kcal/mol to a constant value of 11.2 kcal/mol. Starting with only ADP present, the $\Delta G_p$ rose from approximately 9.0 kcal/mol to a

![Fig. 1. Oxygen electrode recordings illustrating control of respiration by $\Delta G_p$. Respiration was measured at 36° in a medium containing sucrose (250 mM), Na$^+$/Hepes (10 mM), MgCl$_2$ (5 mM), poly-L-lysine (20 μg), ETP$_c$ (0.64 mg), and different concentrations of Na$^+$/P as indicated in a volume of 1.25 ml at pH 7.5. One minute after the initiation of respiration with NADH (0.6 mM), mixtures of ATP plus ADP at different ATP/ADP ratios as indicated were added by a single addition to give a final adenine nucleotide concentration of 2 mM.](http://www.jbc.org/)

![Fig. 2. The effect of $\Delta G_p$ on respiration. Respiration was measured at 36° as described for Fig. 1. For experiments designated by ●, 5 mM Na$^+$/P, was present and the ATP/ADP ratio was varied from 0.01 to 100. For the experiments designated by ▲, the $P_i$ concentration was varied from 0.5 to 10 mM while the ATP/ADP ratio was kept constant at 1:1. For the experiments designated by ■, the $P_i$ concentration was varied from 0.1 to 10 mM while the ATP/ADP ratio was kept constant at 1:9. --- indicates the respiratory rate recorded before the addition of adenine nucleotides. The respiratory rate in the presence of the uncoupler carbonyl cyanide m-chlorophenylhydrazone (1 μM) was 1300 ng atoms of oxygen/min/mg.)](http://www.jbc.org/)

![Fig. 3. The effect of $\Delta G_p$ on the reduction of NAD$^+$ by succinate. The energy-linked reduction of NAD$^+$ by succinate was measured at 36° in a medium containing sucrose (250 mM), Na$^+$/Hepes (10 mM), MgCl$_2$ (5 mM), NAD$^+$ (1 mM), Na$^+$/succinate (5 mM), ETP$_c$ (0.21 mg), and antimycin (0.21 μg) in a volume of 3 ml at pH 7.5. Mixtures of ATP plus ADP (1 mM total concentration) at ATP/ADP ratios of 1:9, 1:1, and 9:1 were added by a single addition to initiate the reaction. The Na$^+$/P concentration was 0.5 mM (●), 9 mM (▲), or 10 mM (■). --- indicates the rate of NAD$^+$ reduction driven by phenazine methosulfate (PMS) (2 μM) plus ascorbate (10 mM) measured in a similar medium without added ATP, ADP, or P. The reported rates have been corrected for antimycin-insensitive NADH oxidation by adding on the rate of NADH oxidation which was observed following the addition of carbonyl cyanide m-chlorophenylhydrazone (1 μM) after 2 min reaction time.)](http://www.jbc.org/)
value of 11.6 kcal/mol. In both cases 2 to 3 min were required in order to establish a constant $\Delta G_p$. These steady state values of $\Delta G_p$ achieved from the initial presence of either ATP or ADP agree well and indicate that the $\Delta G_p$ produced by NADH respiration corresponded to about 11.4 kcal/mol for this ETP preparation.

**DISCUSSION**

The direct chemical determination of the $\Delta G_p$ normally produced by NADH respiration (11.4 kcal/mol, Fig. 4) agrees reasonably well with the indirect estimates from null point experiments (average 10.7 kcal/mol). The null point experimental design gives a value of the $\Delta G_p$ that is a characteristic of the mechanism of oxidative phosphorylation in these inverted vesicles and is not simply a result of membrane damage or "loose coupling." The high degree of stimulation of respiration by uncouplers as well as the stimulation by ADP much in caecox of that previously observed for submitochondrial particles (26) indicates that ETPs are in fact "tightly coupled." The finding that similar values of the characteristic $\Delta G_p$ are determined from studies of either respiration or reverse electron transfer indicates that the characteristic $\Delta G_p$ is approached by electron transfer in either direction. A similar characteristic value, 10.2 kcal/mol, has been reported for the $\Delta G_p$ formed during succinate oxidation by submitochondrial vesicles prepared by sonication of rat liver mitochondria (27). The latter vesicles, however, do not exhibit significant respiratory control (27).

The value of $\Delta G_p$ characteristic of oxidative phosphorylation in inverted submitochondrial particles, approximately 11 kcal/mol, is similar to values measured for the internal compartment of intact mitochondria. Values of 11.2 to 11.8 kcal/mol (6), 11.8 kcal/mol (14), and 11.6 kcal/mol (8) have been reported for the intramitochondrial $\Delta G_p$ of rat liver. As shown in the following paper, the electrochemical proton gradient of submitochondrial particles also responds to changes in $\Delta G_p$. Accordingly, a $\Delta G_p$ of 11 kcal/mol would correspond to an electrochemical proton gradient equivalent to approximately 240 mV, based on a stoichiometry of 2H+/ATP for the reversible ATPase. Such a stoichiometry has been measured during the hydrolysis of ATP by both submitochondrial particles (20) and mitochondria (28, 29). A value of 240 mV is also similar to values of the electrochemical proton gradient measured in intact mitochondria by different techniques (2, 7).

The higher external $\Delta G_p$ values, 15 to 16 kcal/mol, observed in suspensions of intact mitochondria (6, 8, 12-14) are apparently a consequence of the molecular mechanisms of the adenine nucleotide and phosphate transport systems (15-17). Electrogenic transport of ATP outwards in exchange for ADP driven by the membrane potential (30) and electroneutral phosphoric acid transport caused by the pH gradient (31, 32) can account for the observed values of the extramitochondrial $\Delta G_p$ (17). The measured difference between the $\Delta G_p$ values of the internal and external compartments ($\Delta \Delta G_p$), approximately 4 kcal/mol (6, 8, 14), would be accommodated by an electrochemical proton gradient ($\Delta \mu_{H+}$) of about 170 mV since such a mechanism involves the electrogenic transport of one proton and $\Delta G_p = nF \Delta \mu_{H+}$. Alternatively, a $\Delta \mu_{H+}$ of 240 mV could support a $\Delta \Delta G_p$ corresponding to about 5.5 kcal/mol.

In regard to the control of electron transfer, the finding that the $\Delta G_p$ can modulate the rate of respiration in submitochondrial particles is consistent with observations that the external $\Delta G_p$ can regulate the rate of respiration of intact mitochondria (6, 8, 9, 11). Because the internal and external $\Delta G_p$ are interrelated through the electrochemical proton gradient as a consequence of the mechanisms of adenine nucleotide and phosphate transport, either or both will change in response to metabolic perturbations.

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