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THERMODYNAMICS OF REVERSE ELECTRON TRANSFER ACROSS SITE 1: ATP/2e$^-$ IS GREATER THAN ONE

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Summary: ATP-driven, rotenone-sensitive, reverse electron transfer from succinate to acetoacetate was measured in rat liver mitochondria in the presence of cyanide. In the approach to equilibrium, the absolute ratio of the free energy change of electron transfer to that of ATP hydrolysis exceeded 1, tending towards about 1¼. The data support an $H^+/2e^-$ stoichiometry of 5 for Site 1 as predicted by a thirteen-proton model of chemiosmotic coupling.

In mitochondrial oxidative phosphorylation, electron transfer reactions provide free energy to drive phosphorylation of ADP at three 'coupling sites' - Sites 1, 2 and 3. Although most agree that the high energy intermediate between electron transfer and phosphorylation is $\Delta\tilde{\mu}_{H^+}$,[1] the stoichiometries of processes which generate and consume $\Delta\tilde{\mu}_{H^+}$ are in dispute. As a result, several schemes of chemiosmotic coupling are under active consideration which differ as to fundamental ATP/O and ATP/site stoichiometries [cf. 1].

Recently, this laboratory has reevaluated the ATP/O and ATP/site ratios of the forward reactions of mitochondrial oxidative phosphorylation [1-3]. Analysis of ATP to oxygen flux ratios during state 3 and free energy force ratios during state 4 suggested a thirteen-proton model of chemiosmotic coupling in which $H^+/2e^-$ ratios are 5, 4 and 4 and ATP/2e$^-$ ratios are 1¼, ½ and 1½, respectively, for Sites 1, 2 and 3. The thirteen-proton model differs from other chemiosmotic schemes in predicting an ATP/2e$^-$ stoi-

[1] **Abbreviations:** AcAc, acetoacetate; CCCP, carbonyl cyanide m-chlorophenylhydrazone; DCIP, 2,6-dichlorophenol-indophenol; EDTA, ethylenediaminetetraacetic acid; ΔG_P, phosphorylation potential or negative free energy change of ATP hydrolysis; ΔG_R, redox potential or free energy change of two-electron transfer; HEPES, N-2-hydroxyethylpiperazine-N-2-ethane-sulfonic acid; 3-OH-B, 3-hydroxybutyrate; Mal, malate; SMP, submitochondrial particles; Succ, succinate; Tris, tris(hydroxymethyl)aminomethane; $\Delta\tilde{\mu}_{H^+}$, the proton electrochemical potential gradient.

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chiometry for Site 1 of more than 1. In this communication, we follow the thermodynamics of ATP-driven reverse electron transfer across Site 1 in the approach to equilibrium. We find that the free energy change of the driven reaction (electron transfer) exceeds the negative free energy of the driving reaction (ATP hydrolysis). This finding indicates that ATP/$2e^-$ for Site 1 is greater than 1.

Methods

Incubation and Acid Extraction - Rat liver mitochondria, 2.5 mg/ml of protein, were incubated in 150 mM sucrose, 1 mM Na_2EDTA, 1 mg/ml bovine serum albumin (essentially fatty acid free), 1 mM disodium succinate, 1 mM freshly dissolved lithium acetoacetate, 2 mM freshly neutralized KCN, and 25 mM K-HEPES buffer, pH 7.4, 23°C. Reverse electron transfer was initiated with 5 mM ATP. Controls without ATP or with of 2 µM rotenone were run in parallel. Aliquots of 5 ml were quenched in 2.5 ml cold 2N perchloric acid, neutralized, and frozen for later analysis [1,4]. For the zero-time sample, an aliquot was quenched before ATP addition. ATP was then added to the acidified suspension.

Metabolite Determinations - All determinations were in duplicate or triplicate. 3-Hydroxybutyrate, acetoacetate (within 24 hours), ATP, ADP, AMP, and Pi were determined as described [1]. Malate was assayed with malate dehydrogenase [4]. Succinate was determined using SMP for succinate dehydrogenase. SMP (1mg/ml protein) initiated the reaction in 2 µM rotenone, 0.15 µg/ml antimycin, 1 µM oligomycin, 2 mM KCN, 2 µM CCCP, 80 mM tris-Cl buffer, pH 8.0, 23°C, and DCIP (Sigma Chemical Co., St. Louis, Mo.) to an absorbance of 1.1 to 1.3 at 600 nm (nominally 100 µM). Absorbance changes were corrected for turbidity of SMP, and succinate concentrations were read from a standard curve.

Membrane Isolations - Rat liver mitochondria were isolated in 0.25 M sucrose, 2 mM K-HEPES buffer, pH 7.4 [1]. SMP were prepared as described [5] with the exception that frozen-thawed rat liver mitochondria replaced mitoplasts and at least 3 times the sonic energy per mg protein was used.

Free Energy Calculations - The negative free energy change of ATP hydrolysis, ΔG_P, was calculated using a computer program [6] correcting for fluctuations in pH and temperature and using Rosing and Slater's [7] value for $\Delta G_P^{\circ\prime}$. An ionic strength of 0.1 M was assumed.

For the electron transfer reaction:

$$\text{Succinate} + \text{acetoacetate} \Longleftrightarrow \text{malate} + \text{3-hydroxybutyrate} \qquad (1)$$

the free energy change, ΔG_R, is:

$$\Delta G_R = \Delta G_R^{\circ\prime} + 1.36 \log \frac{[\text{Mal}][\text{3-OH-B}]}{[\text{Succ}][\text{AcAc}]} \qquad (2)$$

where:

$$\Delta G_R^{\circ\prime} = -nF\Delta E_m + \Delta G^{\circ\prime}_{fum} = 12.82 \text{ kcal/mol} \qquad (3)$$

ΔE_m is the difference in E_m at pH 7 of the 3-hydroxybutyrate/acetoacetate (E_m = -0.266 V [8]) and succinate/fumarate (E_m = 0.031 V [9]) couples. $\Delta G^{\circ\prime}_{fum}$ for the fumarase reaction is -0.88 kcal/mol [10]. **F** is Faraday's constant and n equals 2.

Results

Addition of ATP to rat liver mitochondria incubated with succinate, acetoacetate and cyanide led to oxidation of succinate to malate, reduction of acetoacetate to 3-hydroxybutyrate, and hydrolysis of ATP to ADP and Pi (Figure 1). This reverse electron transfer was ATP-dependent and blocked by rotenone, a specific inhibitor of electron transfer through Site 1. Fumarate did not accumulate, principally because $\Delta G^{\circ\prime}_{fum}$ favors malate formation [10], and fumarate, unlike malate, is not readily transported across the inner membrane [11].

More acetoacetate was reduced than succinate oxidized. Klingenberg and von Häfen [12] showed that this inequality was abolished in the absence of oxygen. Thus, in the presence of oxygen, electrons from succinate were supplemented by an endogenous

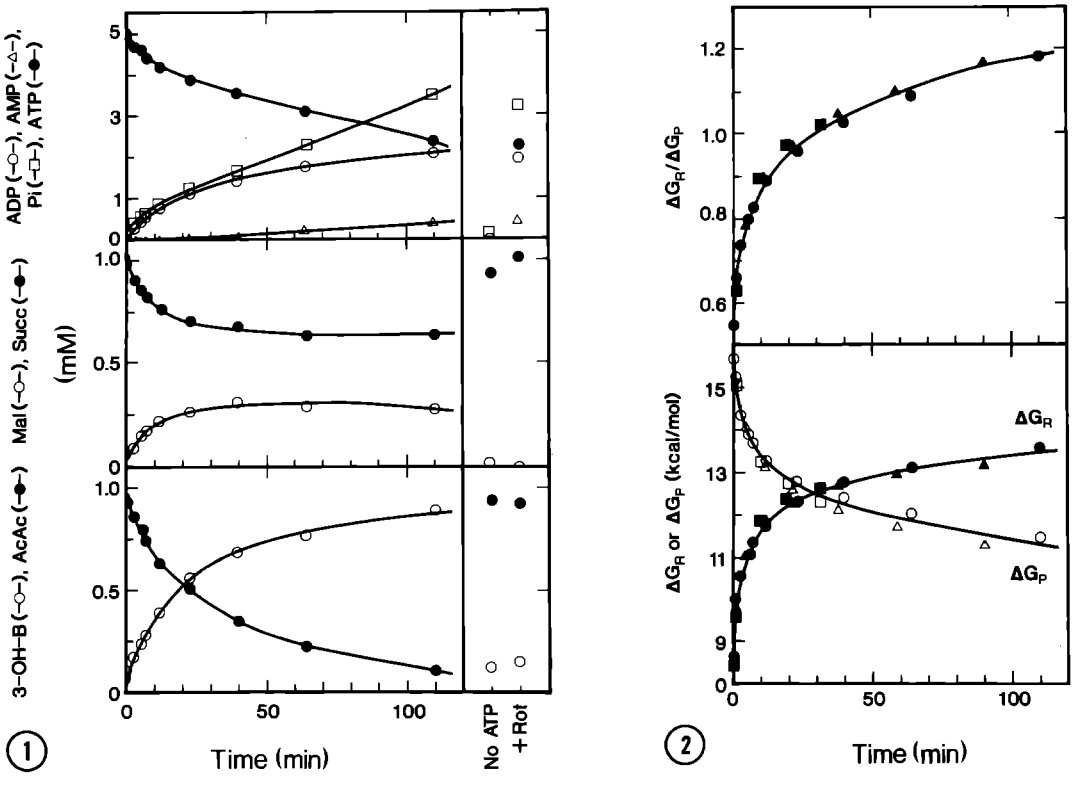

Figure 1. **Metabolite concentrations during ATP-driven reverse electron transfer.** Rat liver mitochondria were incubated with succinate, acetoacetate and KCN, and the reaction was initiated with ATP (zero time) as described in Methods. Panels to the right are control incubations for 110 minutes in the absence of ATP (No ATP) or in the presence of 2 μM rotenone (+Rot).

Figure 2. **ΔG_P, ΔG_R, and $\Delta G_R/\Delta G_P$ during ATP-driven reverse electron transfer.** The symbols represent three different experiments. Conditions as described in Figure 1.

substrate via an oxygen-dependent pathway, e.g. lipid peroxidation. Since electron transfer was ATP-dependent and rotenone-sensitive, the endogenous substrate must feed into the electron transfer sequence at a level at least as electropositive as ubiquinone.

During ATP-driven reverse electron transfer, ΔG_P decreased from more than 15 kcal/mol to less than 12 kcal/mol, ΔG_R increased from less than 9 kcal/mol to more than 13 kcal/mol, and the force ratio, $\Delta G_R/\Delta G_P$, increased to 1.18 (Figure 2). From equilibrium thermodynamics,

$$\Delta G_R/\Delta G_P \leq n \qquad (4)$$

where n is the mechanistic ATP/$2e^-$ stoichiometry. Thus, n is greater than or equal to 1.18.

Discussion

Depending on the proton ratios for the F_1F_0-ATPase, for the protein complexes of the respiratory sequence, and for the metabolite transport systems, all of which are now in dispute, various models of chemiosmotic coupling have been proposed [cf. 1]. The findings here support a thirteen-proton model of chemiosmotic coupling [2,3] in which the ATP/$2e^-$ stoichiometry for Site 1 is 5/4 - the quotient of 5 protons translocated at Site 1 ($H^+/2e^-$) and 4 protons required for ATP synthesis and transport. While the results also appear to support Wikström's [13] prediction of an ATP/$2e^-$ of 4/3 for Site 1, this value and Wikström's ten-proton scheme are based in part on the assumption that Sites 1 and 2 combine to an ATP/$2e^-$ of 2, a stoichiometry inconsistent with recent thermodynamic evidence that shows ATP/$2e^-$ for Sites 1+2 cannot exceed 1.8 [1].

During ATP-driven reverse electron transfer, the mechanistic ATP/$2e^-$ stoichiometry must always exceed $\Delta G_R/\Delta G_P$. ΔG_R and ΔG_P are state functions which express the energetic poise of electron transfer and ATP hydrolysis. Considerations of pathway or mechanism, for example the concentrations of metabolic intermediates or the contributions of endogenous substrates to electron flux, are not relevant to the computation of ΔG_R and ΔG_P. As logarithmic functions, ΔG_R and ΔG_P are relatively insensitive to errors in metabolite measurements. A greater than 15 fold change in a metabolite concentration would be required to decrease ΔG_R or increase ΔG_P such that they are

equal in the approach to equilibrium. Alternatively, $\Delta G_P^{o'}$ must be 1.7 kcal/mol larger or ΔE_m 0.05 V smaller. Errors of this magnitude are unlikely.

Four other studies have presented data open to thermodynamic evaluation of the stoichiometry across Site 1. Klingenberg and von Häfen [12] described ATP-driven reverse electron transfer in rat liver mitochondria but did not allow the reaction to approach equilibrium. Their experiments were terminated as $\Delta G_R/\Delta G_P$ calculated from their data reached about 1, and the ratio was still rising too rapidly to extrapolate to a maximal value in the approach to equilibrium.

De Jonge and Westerhoff [14] observed an $H^+/2e^-$ of 3 in SMP from the ratio of ΔG_R to $\Delta\tilde{\mu}_{H^+}$. However, Berry and Hinkle [15] recently showed that $\Delta\tilde{\mu}_{H^+}$ can be overestimated by as much as 50% unless careful corrections are made for nonspecific binding of permeant ions. Thus, $\Delta G_R/\Delta\tilde{\mu}_{H^+}$ in De Jonge and Westerhoff's work may be substantially underestimated.

In beef heart SMP, Rottenberg and Gutman [16] reported a value for $\Delta G_R/\Delta G_P$ of 1.35 as equilibrium was approached during ATP-driven reverse electron transfer across Site 1. Scholes and Hinkle [17] recently reported a ratio of 1.4 in similar experiments. ATP stoichiometries should be greater in SMP than in intact mitochondria because $\Delta\tilde{\mu}_{H^+}$ is not required for transport of ATP, ADP and Pi. Indeed, because of loose coupling in SMP, the mechanistic $ATP/2e^-$ for Site 1 is likely to be substantially higher $\Delta G_R/\Delta G_P$, probably closer to 5/3 as predicted by the thirteen proton model [cf. 3].

Overall ATP/O stoichiometries will also be greater in SMP than in mitochondria. The thirteen-proton model and Wikström's ten-proton model predict ideal NAD-linked ATP/O ratios of $4\frac{1}{3}$ and 5, respectively. However, $-\Delta G_R/\Delta G_P$ during NADH-supported oxidative phosphorylation by SMP decreases to 4.7 [5,18]. This value is an upper limit to the ATP/O stoichiometry. Again because of loose coupling, the true stoichiometry is probably closer to 4 consistent with the thirteen-proton model.

Thus, analysis of the literature suggests that the $ATP/2e^-$ stoichiometry of Site 1 has been underestimated. The thermodynamic evidence presented here establishes that this stoichiometry exceeds 1 and approximates $1\frac{1}{4}$ as predicted by a thirteen-proton chemiosmotic scheme for mitochondrial oxidative phosphorylation.

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