NEAR THERMODYNAMIC EQUILIBRIUM OF OXIDATIVE PHOSPHORYLATION BY INVERTED INNER MEMBRANE VESICLES OF RAT LIVER MITOCHONDRIA

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1. Introduction

Homogenously inverted vesicles prepared from purified mitochondrial inner membranes generate during oxidative phosphorylation a steady state phosphate potential (ΔG_P)* of 9.8–10.5 kcal/mol [1,2]. This value is 30–40% less than the steady state ΔG_P generated by intact mitochondria [3]. In [4] it was suggested that the larger steady state ΔG_P maintained by intact mitochondria resulted from the operation of the ADP^{3-}–ATP^{4-} and P_i^-–OH^- carriers which together provide a $\Delta\tilde{\mu}_{H^+}$-dependent mechanism for actively pumping ADP and P_i into and ATP out of the mitochondrial matrix space. In addition, it was shown that one of these carriers, the ADP–ATP carrier, is rate limiting for the overall reaction of oxidative phosphorylation by intact mitochondria [4]. As pointed out in [5], this latter observation suggests that for intact mitochondria the reactions of electron transport and internal (matrix) ATP synthesis are at near thermodynamic equilibrium.

If the preceding considerations are correct, then the following should apply for oxidative phosphorylation by inverted inner membrane vesicles:

(1) Since ATP synthesis by inverted vesicles does not require ATP, ADP and P_i translocation, the proton whose transmembrane movement ordinarily drives this translocation will be available to drive other energy requiring reactions such as ATP synthesis. Thus, the ATP/O ratio for inverted vesicles should be higher than for intact mitochondria. The magnitude of difference will depend upon the stoichiometry of proton translocation in the respiratory chain.

2. If the reactions of electron transport and internal ATP synthesis are at near equilibrium for intact mitochondria, then inverted membrane vesicles should be able to maintain the bulk aqueous reactants of electron transport and oxidative phosphorylation at or close to thermodynamic equilibrium.

Here, preliminary experiments examining these two predictions are presented.

* Where $\Delta G_P = \Delta G_P^\circ + 1.36 \log \frac{[ATP]}{[ADP]\,[P_i]}$

2. Methods

Membrane vesicles were prepared by mild sonication of purified inner membranes using a slight modification of the procedure in [1,6]. The purified inner membrane-matrix (mitoplast) fraction [7] was washed 3 times in 7.5-fold diluted, BSA free isolation medium and resuspended in water to 12 mg protein/ml. This was sonicated for 2–3 min at 0°C in 15 s bursts using the microprobe of a Model 185 Branson Cell Disrupter at a power output of 30 W. The sonicated suspension was then centrifuged at 9000 × *g* for 15 min to remove undisrupted membranes, and the supernatant was further centrifuged at 144 000 × *g* for 1 h. The resulting pellet was resuspended in 0.25 M sucrose to a 50 mg protein/ml and was used immediately or after storage at −75°C. The vesicular membranes were virtually 100% inverted as judged by morphological [6], metabolic [2,6] and immunochemical [8] criteria. As assayed in the reaction medium in fig.1, respiration by inverted vesicles was inhibited 30–50% by oligomycin. The ratio of uncoupler stimulated to

oligomycin inhibited respiration ranged from 5–8.

ATP concentration was measured continuously with firefly luciferase bioluminescence [1,2,9]. All quantitative ATP determinations were made by addition of ATP standard. ΔG_P was calculated using a standard free energy, ΔG_P°, of 7.2 kcal/mol [10]. Oxygen was measured polarographically [11]. Actual concentrations of stock ATP, ADP, NADH and NAD solutions were determined spectrophotometrically.

3. Results

ATP synthesis by inverted vesicles became activated during the cycle of ATP synthesis and hydrolysis that followed the addition of a small (106 μM) pulse of NADH (fig.1a). This activation was evident because,

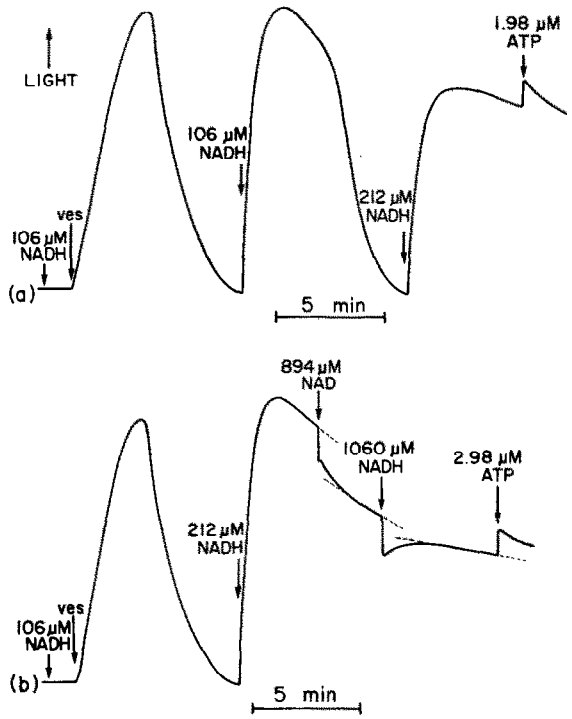

Fig.1. ATP synthesis during oxidative phosphorylation by inverted inner membrane vesicles. Reaction medium was 150 mM sucrose, 5 mM $MgCl_2$, 10 mM KP_i buffer, 16 mM potassium *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (K-Hepes) buffer (pH 7.4), 19.5 μM ADP, 7.1 μM luciferin, 10 units/ml luciferase, 0.2 mg protein/ml inverted vesicles, 23° C. NADH, NAD and ATP were added in the amounts indicated. In (b), the dotted lines extrapolate the nearly linear decrease of luminescence occurring at steady state. This decrease is due in part to product inhibition of the luminescence reaction [1,9].

after a second pulse of NADH, ATP synthesis was considerably more rapid. Moreover, a steady state ATP level was rapidly achieved during which little net ATP synthesis or hydrolysis occurred. Upon exhaustion of the second NADH pulse, ATP was again hydrolyzed. A third NADH pulse demonstrated that there was no further apparent activation of ATP synthesis. In good agreement with studies employing succinate as respiratory substrate [1,2], ATP/ADP was 1.3 at the end of the experiment, as determined after the addition of ATP standard.

The steady state ATP to ADP ratio maintained after activation was dependent upon the NADH/NAD ratio, since NADH and NAD increased and decreased, respectively, the steady state concentrations of ATP. As shown in fig.1b, both NADH and NAD caused a rapid, stepwise decrease in the light signal. This was due to direct, ionic inhibition of the luminescence reaction [12]. Subsequently, the light signal recovered after NADH, but continued to decrease after NAD until a new steady state was reached. Since control experiments (data not shown) in the absence of vesicles revealed only rapid, stepwise decreases in luminescence after NADH or NAD, the slow recovery of the light signal after NADH must indicate an increase in ATP/ADP, while the continued decrease of light after NAD must indicate a drop in ATP/ADP. Changes in respiratory rate did not cause these alterations in ATP/ADP, since parallel oxygen electrode experiments determined that respiratory rate was zero order with respect to NADH and was not inhibited by NAD.

In order to investigate in greater detail the relation of ATP/ADP to NADH/NAD, inverted vesicles were first activated with 106 μM NADH. After the NADH was exhausted, luciferase and variable amounts of NAD were added. At the end of 15 min total incubation, another pulse of NADH was added. Finally, 4 min later, ATP concentration was quantified by addition of ATP standard. Each experiment was repeated at 3 different P_i concentrations. NADH plus NAD was kept constant at ~1 mM, and final NADH and NAD concentrations were corrected for NADH oxidation. ADP concentration was inferred from ATP concentration since total adenine nucleotide was constant. As shown in [2], adenylate kinase activity was negligible in these membrane preparations so that AMP formation by this mechanism was insignificant, and virtually all adenine nucleotide was in the form of either ADP or ATP. With the values of ATP, ADP, and P_i, ΔG_P was calculated and plotted versus $-\Delta G_R$,

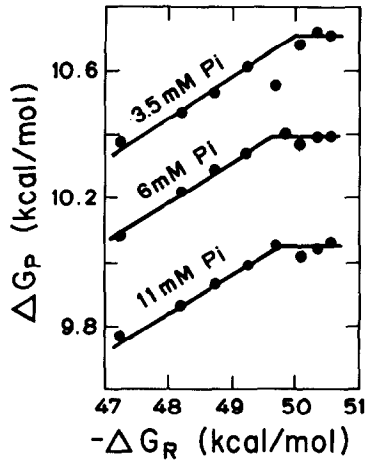

Fig.2. ΔG_P as a function of $-\Delta G_R$. Reaction medium was as in fig.1, except that 10 mM Na-Hepes replaced K-Hepes and KP_i was varied as indicated. Total pyridine nucleotide (NADH + NAD) ranged from 0.98–1.04 mM. See text for order of additions.

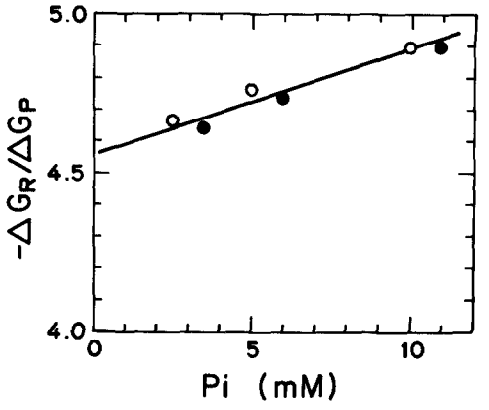

Fig.3. $-\Delta G_R/\Delta G_P$ as a function of P_i. Filled circles are from experiments of fig.2. Open circles are from a second experimental series in a reaction medium identical to fig.1, except that changes in KP_i were compensated by equimolar changes in K-Hepes.

the redox potential, which is here defined as:

$$\Delta G_R = \Delta G_R^\circ + 1.36 \log \frac{[NAD]}{[NADH]\ [O_2]^{1/2}} + 1.36\ (pH - 7)$$

Oxygen was assumed to be constant at 200 μM. ΔG_R° = −52.7 kcal/mol and is the standard free energy at pH 7 of the reaction:

$$NADH + H^+ + \tfrac{1}{2}O_2 \rightarrow NAD^+ + H_2O$$

At constant P_i, ΔG_P was a biphasic function of ΔG_R (fig.2). At values of ΔG_R less negative than ~−49.7 kcal/mol, ΔG_P was a linear function of ΔG_R, but at values of ΔG_R more negative than 49.7 kcal/mol, ΔG_P was constant and independent of changes in ΔG_R. At all values of ΔG_R, ΔG_P increased as P_i decreased.

If the phosphorylating system and the respiratory system are at thermodynamic equilibrium, and if their coupling efficiency is 100%, then the $-\Delta G_R/\Delta G_P$ ratio will equal the stoichiometry of ATP synthesis, i.e., the number of ATP molecules formed for each substrate molecule oxidized. However, it is likely that there are competing ATPase reactions which will tend to hold the coupled reaction away from equilibrium. This view is supported by the observation that ΔG_P increased as P_i decreased. In fig.3, $-\Delta G_R/\Delta G_P$ is a linear function of P_i and approaches 4.5 as P_i goes to zero.

4. Discussion

The observation that ΔG_P is a linear function of ΔG_R (fig.2) is consistent with the conclusion that under coupled, steady state conditions, the reactions of respiration and of ATP synthesis are close to thermodynamic equilibrium. That these reactions are not in complete equilibrium is indicated by the fact that ΔG_P increases as P_i decreases. This latter observation suggests that ATPase activity by a subpopulation of uncoupled vesicles may be shifting the overall reaction of oxidative phosphorylation away from full thermodynamic equilibrium.

Another notable feature of the plot of ΔG_P versus $-\Delta G_R$ is the abrupt leveling off of the curves at ΔG_R values more negative than −49.7 kcal/mol. Assuming a maximal $-\Delta G_R/\Delta G_P$ ratio of 4.5, then the maximal ΔG_P generated by inverted vesicles is 11 kcal/mol. The leveling off of these curves resembles the breakdown of membrane resistance observed [13] at membrane potentials ($\Delta\Psi$) more negative than −200 mV. If this view is correct, then $\Delta\Psi$ decreases proportionately with ΔG_R until it is ~−200 mV. At this point membrane resistance falls abruptly, and further decreases in ΔG_R cause no further decrease in $\Delta\Psi$. If ΔG_P is in equilibrium with $\Delta\tilde{\mu}_{H^+}$, and if $\Delta\Psi$ is a constant and predominant component of $\Delta\tilde{\mu}_{H^+}$, then ΔG_P itself reaches a maximum value and does not increase with further decreases in ΔG_R°.

These findings have special relevance to recent controversies concerning the proton stoichiometry of

Table 1
Several proposed stoichiometries of oxidative phosphorylation by intact mitochondria and inverted inner membrane vesicles

Membrane	H^+/site	H^+/ATPase	H^+/exchange[a]	ATP/O[b]
Intact[c]	2	2	0	3
Inverted	2	2	0	3
Intact[d]	2	2	1	2
Inverted	2	2	0	3
Intact	3	2	1	3
Inverted	3	2	0	4.5
Intact[e]	4	3	1	3
Inverted	4	3	0	4

[a] Of ADP and P_i for ATP
[b] $= \frac{3 \times H^+/\text{site}}{H^+/\text{ATPase} + H^+/\text{exchange}}$
[c] Original chemiosmotic formulation [19]
[d] Proposal in [18]
[e] Proposal in [16]

oxidative phosphorylation (table 1). At least 3 and probably 4 protons per coupling site are ejected from the mitochondrion during respiration [14–16, cf. 17]. One of these protons is expended during ATP, ADP, and P_i translocation, and the remainder is utilized for ATP synthesis. Assuming that the proton stoichiometry of the energy transducing ATPase is one less than the proton/site ratio, this gives rise to an overall ATP/O ratio of 3 for NADH-linked substrates. While accepting the need for a proton for ATP, ADP, and P_i translocation, it is maintained [18] that a proton stoichiometry of 2 H^+/site and 2 H^+/ATPase as originally postulated [19] is correct. The latter workers have presented evidence instead that $\Delta\tilde{\mu}_{H^+}$ coupled translocation of nucleotides and P_i causes a reduction of mitochondrial ATP/O ratios to 2/3rds of classical values.

It is clear that in order to catalyze oxidative phosphorylation, inverted inner membrane vesicles do not require ATP, ADP and P_i translocation. Therefore, an extra proton/coupling site should be available to drive ATP synthesis with the consequence that ATP/O ratios will be greater for inverted vesicles than for intact mitochondria (table 1). At true equilibrium, $-\Delta G_R/\Delta G_P$ will equal ATP/O. In the present study, $-\Delta G_R/\Delta G_P$ approached 4.5 as a limiting value. This is consistent with a proton/site ratio of 3 and a proton/ATPase ratio of 2. However, considering that the inverted vesicles may be somewhat removed from full equilibrium, a proton/site stoichiometry of 4 cannot be definitely excluded, in which case the predicted ATP/O ratio (and limiting $-\Delta G_R/\Delta G_P$ ratio) will be 4. The data reported here are least consistent with a proton/site stoichiometry of 2 which predicts that inverted vesicles at near equilibrium will generate a ΔG_P of 15.5–16.5 kcal/mol, a prediction which exceeds the experimental findings by 50%. Although these preliminary experiments have been discussed primarily in thermodynamic terms, alternate kinetic models may apply (cf. [20]) and further study will be needed.

5. Conclusion

Irrespective of interpretation, the following empirical findings are emphasized for oxidative phosphorylation by inverted inner membrane vesicles of rat liver mitochondria oxidizing NADH:

(1) At constant P_i, ΔG_P was a linear function of ΔG_R for values of ΔG_R less negative than ~–49.7 kcal/mol. For values of ΔG_R more negative than –49.7 kcal/mol, ΔG_P was constant.
(2) For all values of ΔG_R, ΔG_P increased as P_i decreased.
(3) The limiting value of $-\Delta G_R/\Delta G_P$ was 4.5 as P_i approached zero.

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