The phosphate potential maintained by mitochondria in State 4 is proportional to the proton-motive force

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Evidence is presented for a proportional relationship between the extramitochondrial phosphate potential (ΔG_p^{ex}) and the proton-motive force ($\Delta\tilde{\mu}_{H^+}$) across the mitochondrial membrane in rat-liver mitochondria oxidising succinate in State 4, when $\Delta\tilde{\mu}_{H^+}$ is varied by addition of uncouplers or malonate. This relationship was found when precautions were taken to minimise interference with the determination of ΔG_p^{ex} and $\Delta\tilde{\mu}_H$ by intramitochondrial nucleotides, adenylate kinase activity, the quenching method, and $\Delta\tilde{\mu}_H$ -dependent changes in matrix volume. A non-proportional $\Delta G_p^{ex}/\Delta\tilde{\mu}_{H^+}$ relationship was obtained when these precautions were omitted. Our results do not support mosaic protonic coupling, but are not necessarily in conflict with other localised coupling schemes.

Mitochondria *Energy transduction* *Localised chemiosmotic coupling* *Proton-motive force* *Phosphate potential* *ATP synthase stoichiometry*

1. INTRODUCTION

The central tenet of Mitchell's chemiosmotic theory [1], i.e., the involvement of a proton electrochemical potential difference across the energy-transducing membrane ($\Delta\tilde{\mu}_{H^+}$) as the energy-rich intermediate in oxidative and photophosphorylation, is widely accepted but the precise mechanism of membrane-linked energy transduction is still an area of controversy.

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Abbreviations: DNP, 2,4-dinitrophenol; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; S13, 5-chloro-3-*tert*-butyl-2'-chloro-4'-nitrosalicylanilide; AP_5A, P^1,P^5-di(adenosine-5'-)pentaphosphate; ΔG_p^{ex}, extramitochondrial phosphate potential; $\Delta\tilde{\mu}_{H^+}$, the electrochemical potential difference for protons between two separate spaces

One of the aspects of Mitchell's theory that has been challenged is the assumption that protons freely equilibrate with the bulk aqueous phase on both sides of the membrane. A number of experimental results seem to be at variance with such a 'delocalised' coupling scheme. The most prominent apparent discrepancy has been that in incubations of mitochondria, after equilibrium for the ATPase reaction has been reached (State 4), a decrease in $\Delta\tilde{\mu}_{H^+}$ which is brought about via addition of either uncouplers or respiratory inhibitors was not associated with a proportional decrease in ΔG_p^{ex}, as was found by our group [2–4] and others [5]. This has led to the conclusion that either the stoicheiometry (i.e., the number of protons pumped per molecule of ATP hydrolysed or synthesized) is not constant at different values for $\Delta\tilde{\mu}_{H^+}$, or that the measured bulk-phase $\Delta\tilde{\mu}_{H^+}$ is not the relevant force as sensed by the ATPase and the adenine nucleotide translocator. The latter conclusion implies that there is a local force (e.g., local $\Delta\tilde{\mu}_{H^+}$) close to the membrane, which differs in

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magnitude from the bulk aqueous phase $\Delta\tilde{\mu}_{H^+}$. This difference can only occur if rapid equilibration between the local coupling site and the bulk phase is prevented by some sort of resistance, while at the same time bulk-phase protons leak through a part of the membrane that is not part of the local coupling site. By adding this assumption to the postulates of the chemiosmotic theory, authors in [6] arrived at their so-called mosaic protonic coupling model.

For the simultaneous measurement of ΔG_p^{ex} and $\Delta\tilde{\mu}_{H^+}$ a number of experimental errors and uncertainties can be foreseen. Factors such as intramitochondrial nucleotides, ATP hydrolysis during and after termination of the reaction and the activity of adenylate kinase could disturb a correct measurement of ΔG_p^{ex}, and $\Delta\tilde{\mu}_{H^+}$-dependent matrix volume changes could interfere with the determination of $\Delta\tilde{\mu}_{H^+}$. We have therefore reinvestigated the relationship between ΔG_p^{ex} and $\Delta\tilde{\mu}_{H^+}$, taking extra precautions to minimise possible sources of error. The results obtained strongly deviate from those reported previously [2–5] in that we now found a proportional relationship between ΔG_p^{ex} and $\Delta\tilde{\mu}_{H^+}$, with a constant stoicheiometric $\Delta G_p^{ex}/\Delta\tilde{\mu}_{H^+}$ ratio of approx. 3 when $\Delta\tilde{\mu}_{H^+}$ was varied using either uncouplers or the respiratory inhibitor malonate.

2. MATERIALS AND METHODS

Rat-liver mitochondria were isolated as in [7], but using 275 mM mannitol/2 mM Hepes (pH 7.0) instead of 250 mM sucrose as the isolation medium. Protein was determined by the biuret method. Oxygen consumption was measured using a Clark oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH). Mitochondria (3.3 mg protein/ml) were incubated at 25°C in a vessel equipped with a stirring device in a medium containing 10 mM succinic acid and 1 mM DL-malic acid. Sodium phosphate, EGTA or EDTA and $MgCl_2$ were present as indicated in the figure legend. The medium was brought to pH 7.0 with NaOH or Tris base and contained mannitol at a final osmolarity of 290 mosM and 500 U catalase/ml (Boehringer, Mannheim, FRG). Rotenone and valinomycin were added. To decrease $\Delta\tilde{\mu}_{H^+}$, uncouplers were used (DNP, FCCP, S13, gramicidin D) or a combination of DNP with the respiratory inhibitor malonate. Finally, ADP or ATP was added. The final concentrations of the added substances and the presence of other additives are given in the figure legend.

Incubations were allowed to last at least 2-times longer than the time needed for coupled mitochondria to reach State 4 respiration. When needed, a few injections of small quantities of H_2O_2 were given to maintain p_{O_2} between 20 and 90% saturation with respect to air. In expt 2 (fig.1A), the total ^3H_2O space and the [^{14}C]mannitol-inaccessible ^3H_2O space (matrix volume, see [8]) were measured after centrifugation of the mitochondria through 500 μl silicone oil (AR200/AR20, 3:1, Wacker GmbH, Munich) for 1 min at 9000 × g. Counts were corrected for quenching and channel cross-talk. In the other experiments, matrix volumes were calculated from the K^+ content relative to fully de-energised mitochondria, assuming a matrix volume of 0.4 μl/mg protein in fully de-energised mitochondria and assuming osmotic equilibrium with a univalent K^+ salt. This method gave results that were in good agreement with the method in which ^3H_2O and [^{14}C]mannitol were used. The samples for the measurement of matrix volumes, $\Delta\tilde{\mu}_{H^+}$ and ΔG_p^{ex} were taken simultaneously. $\Delta\tilde{\mu}_{H^+}$ was calculated from the K^+ and phosphate distributions between inside and outside of the mitochondria after parallel centrifugation of the mitochondria through 500 μl silicone oil into 350 μl of 0.4 M $HClO_4$/20 mM EDTA. ΔG_p^{ex} was calculated from the concentrations of ATP and ADP and P_i in the sample after filtering the sample through micro glass-fibre filters (Whatman, GF/F) and directly quenching the filtrate in an organic quench mixture according to [9], exactly as described in [10]. For comparison, a second method was used omitting the filtration step and using $HClO_4$ (0.4 M $HClO_4$/20 mM EDTA) for quenching.

D-[1-^{14}C]Mannitol (50 μCi/ml) and ^3H_2O (1 mCi/ml) were obtained from The Radiochemical Centre, Amersham, England. DNP ('for synthesis grade') was from Merck-Schuchard, Hohenbrunn, FRG. S13 was a gift from Dr P. Hamm, Monsanto Company, St. Louis, MO. FCCP was a gift from Dr P.G. Heytler and gramicidin D was obtained from Boehringer, Mannheim, FRG.

3. RESULTS

ΔG_p^{ex} and $\Delta\tilde{\mu}_{H+}$ were simultaneously measured in mitochondrial incubations in State 4, with succinate as the substrate for oxidation. Fig.1A represents a summary of 4 different sets of experiments, numbered 1–4. The uncouplers DNP, FCCP, S13 or gramicidin D, or DNP in combination with the respiratory inhibitor malonate, were used at various concentrations to decrease $\Delta\tilde{\mu}_{H+}$. We obtained a linear relationship between ΔG_p^{ex} and $\Delta\tilde{\mu}_{H+}$ that can be extrapolated through zero. Notably, a decrease in ΔG_p^{ex} proportional to the decrease in $\Delta\tilde{\mu}_{H+}$ was obtained, irrespective of

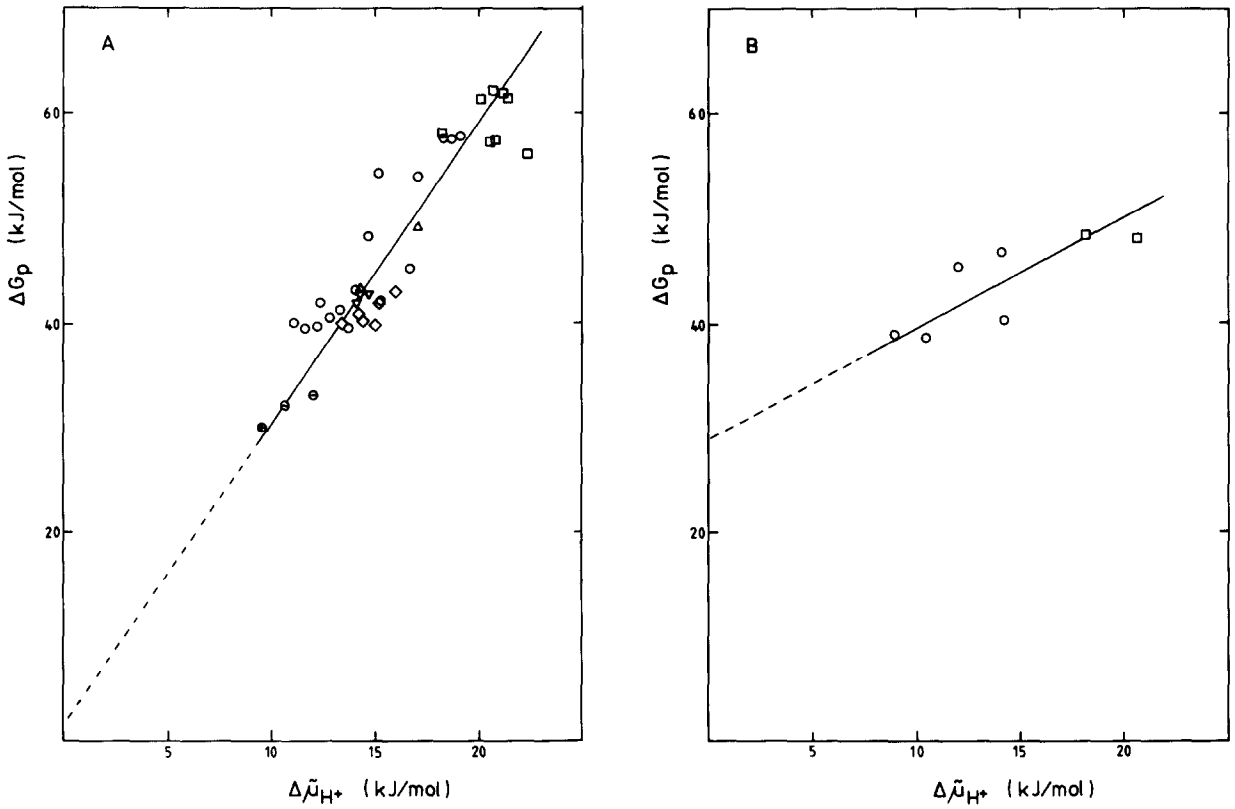

Fig.1. Dependence of extramitochondrial ΔG_p on $\Delta\tilde{\mu}_{H+}$ of mitochondria in State 4 with succinate as substrate. (A) Summary of 4 experiments in which $\Delta\tilde{\mu}_{H+}$ was varied using: (□) no uncoupler; (○) DNP; (⊖ and ⊕) DNP + malonate; (◇) gramicidin D; (△) S13; (▽) FCCP. Final concentrations of additions and constituents of the medium not specified in section 2 were as follows: (Expt 1) 10 mM sodium phosphate, 2 mM EGTA, 0.4 μg rotenone and 0.2 μg valinomycin per mg protein, 1 mM ADP. (□) No uncoupler; (○) 20, 40 or 80 μM DNP. (Expt 2) Conditions as in expt 1. (□) No uncoupler; (○) 80 μM DNP; (⊖) 80 μM DNP + 1 mM malonate + 4 mM $MgCl_2$ + 10 mM AMP; (⊕) 80 μM DNP + 1 mM malonate + 2 mM AP_5A (added as trilithium salt). (Expt 3) 20 mM Mops, 10 mM sodium phosphate, 4 mM EGTA, 0.7 μg rotenone and 0.1 μg valinomycin per mg protein, 5 mM ADP (or as a control 5 mM ATP). (□) No uncoupler; (○) 12, 25, 50 or 100 μM DNP; (◇) 0.3 or 1.0 μg gramicidin per mg protein. (Expt 4) 20 mM Mops, 6 mM sodium phosphate, 2 mM EDTA, 0.7 μg rotenone and 0.1 μg valinomycin per mg protein. (□) No uncoupler; (○) 50 or 150 μM DNP; (△) 0.035 or 0.56 nmol S13 per mg protein; (▽) 0.16, 0.74 or 1.60 nmol FCCP per mg protein. (B) Experiment performed in parallel to expt 1 of A, with the same mitochondrial preparation and under the same conditions, except that 4 mM $MgCl_2$ was added, the samples were not filtered, the samples were quenched in $HClO_4$, only 0.2 mM ADP was added and a constant matrix volume was assumed of 1.0 μg per mg protein. For the calculation of ΔG_p^{ex} values were taken for ΔG_p^{o} of 28.5 kJ/mol when 4 mM Mg^{2+} was added and of 33.1 kJ/mol when no added Mg^{2+} was present (from [11]). The plotted lines were calculated by major axis regression.

which uncoupler had been used. When the ATPase reaction is in equilibrium, ΔG_p^{ex} should be equal to $n\Delta\tilde{\mu}_{H^+}$ (so that $n = \Delta G_p^{ex}/\Delta\tilde{\mu}_{H^+}$; n is the phenomenological stoicheiometry constant, i.e., the number of protons involved in ATP synthesis plus ATP-ADP and P_i translocation). We found a $\Delta G_p^{ex}/\Delta\tilde{\mu}_{H^+}$ ratio of approx. 3 which was constant at decreasing values of $\Delta\tilde{\mu}_{H^+}$.

In the experiments represented in fig.1A, we took precautions to avoid or correct for some factors possibly disturbing a correct determination of ΔG_p^{ex} and $\Delta\tilde{\mu}_{H^+}$, as listed below.

(1) The samples for the determination of ΔG_p^{ex} were rapidly filtered through Whatman GF/F filters immediately before quenching, thus avoiding the presence of intramitochondrial adenine nucleotides (cf. [12]).

(2) To prevent hydrolysis of ATP to ADP, an organic quench mix [9] was used to terminate reactions instead of $HClO_4$.

(3) Adenine nucleotide concentrations were 1 mM or higher (up to 11 mM).

(4) The matrix volume was determined for each separate incubation for the appropriate calculation of the intramitochondrial P_i and K^+ concentrations (P_i and K^+ distributions being taken as measures of ΔpH and $\Delta\psi$, respectively).

(5) Adenylate kinase was either blocked by AP_5A, or allowed to reach equilibrium.

The experiment represented in fig.1B was performed in parallel with expt 1 in fig.1A with the same mitochondrial preparation. The only differences between the two experiments are that the precautions listed in points 1–5 above were not taken. Most strikingly, a non-proportional relationship between ΔG_p^{ex} and $\Delta\tilde{\mu}_{H^+}$, as has been found previously by our group and others [2–5], was now obtained.

4. DISCUSSION

The non-proportional relationship between ΔG_p^{ex} and $\Delta\tilde{\mu}_{H^+}$ in incubations of rat-liver mitochondria in State 4 reported by our group [2–4] and others [5] has always been a strong argument against delocalised chemiosmotic coupling. We now show that a proportional relationship between ΔG_p^{ex} and $\Delta\tilde{\mu}_{H^+}$ was obtained when only a few alterations were made to the methods used. Some factors that could possibly explain the different results are discussed below.

As the exchange of ATP for ADP via the adenine nucleotide translocator is electrogenic with a stoicheiometry of one charge per exchange [13], a $\Delta\psi$ of 15 kJ/mol is expected to give an intramitochondrial ATP/ADP ratio approx. 400-times lower than the extramitochondrial ratio. The total rat-liver mitochondrial adenine nucleotide content was 18.6 ± 1.7 nmol/mg protein (n = 7, not shown). If the samples for the determination of ΔG_p^{ex} are not filtered and if a high protein concentration and a low extramitochondrial nucleotide concentration are used, the values of ΔG_p^{ex} at the highest $\Delta\tilde{\mu}_{H^+}$ values will therefore be seriously underestimated. On the other hand, at very low values of $\Delta\tilde{\mu}_{H^+}$, ΔG_p^{ex} may then be overestimated because of the ATP that is bound to the high-affinity ATP-binding sites on the ATPase.

At high ATP/ADP ratios, it is important to prevent hydrolysis of ATP to ADP during or after quenching of the reaction. Authors in [10] showed that significant hydrolysis of ATP does take place when reactions are terminated in $HClO_4$, leading to an underestimation of ΔG_p^{ex} at high ATP/ADP ratios, whereas no appreciable ATP hydrolysis takes place when the organic quench mix described in [9] is used.

Another source of error may be presented by the enzyme adenylate kinase. A true equilibrium for the ATPase is not reached unless the rate of the adenylate kinase reaction is practically zero, either because the reaction is in equilibrium or because of quantitative inhibition of the enzyme. We found values for ΔG_p^{ex} as low as 30 kJ/mol when either 10 mM AMP was added in the presence of Mg^{2+} to bring the adenylate kinase reaction close to equilibrium and still have a measurable ATP concentration, or when the adenylate kinase activity was blocked with 2 mM AP_5A in the absence of Mg^{2+} (fig.1A). The other low $\Delta\tilde{\mu}_{H^+}$ points in fig.1A may have somewhat overestimated ΔG_p^{ex} values, since adenylate kinase was not fully blocked and could be shown to be out of equilibrium.

An uncertain factor in the determination of $\Delta\tilde{\mu}_{H^+}$ is the mitochondrial matrix volume. Authors in [8] argued that [^{14}C]mannitol rather than [^{14}C]sucrose should be used for a true representa-

tion of extramitochondrial space, and found approx. 1 μg/mg protein bigger matrix volumes when [^{14}C]sucrose was used than with [^{14}C]mannitol. We found matrix volumes of approx. 1 μl/mg protein in coupled mitochondria in State 4, when using [^{14}C]mannitol. A decrease in matrix volumes with decreasing $\Delta\tilde{\mu}_{H^+}$ of up to 0.5 μg/mg protein can be calculated on the basis of the amount of K^+ that is lost, or can be measured using either [^{14}C]sucrose or [^{14}C]mannitol. This volume decrease leads to an overestimation of the $\Delta\tilde{\mu}_{H^+}$ decrease induced by uncoupler if the matrix volume is taken as being constant, as we did in fig.1B. The magnitude of this error depends on the relative change in volume. If the true matrix volume of coupled mitochondria in State 4 is 1 μl/mg protein, a value we found when we used [^{14}C]mannitol, the relative decrease in volume would be up to 50% and the error would then be up to 3.4 kJ/mol.

The proportional relationship between ΔG_p^{ex} and $\Delta\tilde{\mu}_{H^+}$ reported here does not support the model of mosaic protonic coupling [6] for the mitochondrial system, as this model implies a non-proportional relationship. Our results do not necessarily conflict with other 'localised' coupling schemes. Our present results are in full agreement with the chemiosmotic theory [1]. However, a number of observations cannot be explained easily with a delocalised chemiosmotic coupling scheme (review [14]), so that alternative coupling schemes still may be preferred. Further experiments must show how wide the implications of our present results are.

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