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A COMPARISON OF THE PHOSPHORYLATION POTENTIAL AND ELECTROCHEMICAL PROTON GRADIENT IN MUNG BEAN MITOCHONDRIA AND PHOSPHORYLATING SUB-MITOCHONDRIAL PARTICLES

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Summary

The phosphorylation potential (ΔG_p) and the electrochemical proton gradient ($\Delta\bar{\mu}H^+$) normally maintained during respiration or ATP hydrolysis by mung bean hypocotyl mitochondria and phosphorylating sub-mitochondrial particles have been investigated. Phosphorylation potential experiments using safranine and oxonol-VI, as membrane potential markers for mitochondria and sub-mitochondrial particles, respectively, suggest that the 'null point' ΔG_p (i.e., the phosphorylation potential at which no change in optical signal occurred) corresponds to a value of 15.2 ± 0.7 kcal/mol in mitochondria and 11.2 ± 0.3 kcal/mol in sub-mitochondrial particles. The value of $\Delta\bar{\mu}H^+$ generated by the hydrolysis of ATP was estimated using ion distribution techniques. In each case a rapid centrifugation technique was used to separate the organelle from the suspending medium. The total $\Delta\bar{\mu}H^+$ generated in each case was approx. 200 mV being composed of both membrane potential and pH components. A comparison of $\Delta\bar{\mu}H^+$ with ΔG_p indicates that the apparent H^+/ATP ratio in mung bean mitochondria is 3.4 ± 0.2 while in phosphorylating sub-mitochondrial particles it is 2.2 ± 0.1.

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Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; oxonol-VI, bis(3-propyl-5-oxoisoxozol-4-yl)pentamethineoxonol; $\Delta\psi$, membrane potential; ΔpH, pH differential; $\Delta\bar{\mu}H^+$, electrochemical proton gradient or protonmotive force; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

Introduction

It is generally accepted that the energized state of mitochondria is accompanied by the formation of a large electrochemical proton gradient ($\Delta\bar{\mu}H^+$) which is dependent upon mitochondrial metabolism and which responds to changes in the energy potential [1—10] as originally envisaged in the chemiosmotic hypothesis [12,13]. There is also considerable evidence which suggests that this gradient is the obligatory link between respiration and ATP synthesis [1,3,7,14—16]. A consideration of the thermodynamic aspects of oxidative phosphorylation has indicated that respiring mitochondria maintain an external phosphorylation potential (ΔG_p) that is considerably higher than the matrix value [14,17—21]. ΔG_p is the Gibbs free energy of ATP synthesis and is defined by the relationship $\Delta G_p = -\Delta G'_0 + RT \ln([ATP]/[ADP][P_i])$ where $\Delta G'_0$ is the standard free energy of ATP hydrolysis. It has been suggested that this difference may be due to the transport of ATP, ADP, and P_i in and out of the matrix space [22—24]. Evidence in the literature indicates that from a comparison of $\Delta\bar{\mu}H^+$ with the phosphorylation potential, the number of protons translocated per ATP molecule synthesized in intact mitochondria varies from 2 to 5 [1,3—5,9,10,14,21,22,25—34]. In view of the importance of this stoichiometry with respect to the mechanism of ATP synthesis and electron transport, there is a need for additional approaches to this problem using different material. Although this ratio has been extensively analyzed in mammalian tissues, photosynthetic bacteria and chloroplasts, no information is available from plant mitochondria. In view of the close cellular interaction of plant mitochondria with chloroplasts it is important to ascertain the H^+/ATP stoichiometry in these organelles.

The present paper reports measurements of the phosphorylation potential and the electrochemical proton gradient both in intact mung bean mitochondria and phosphorylating sub-mitochondrial particles. Extrinsic probes of membrane potential, namely safranine [6] and oxonol OX-VI [15], have been used in mitochondria and sub-mitochondrial particles respectively to determine the ΔG_p using the null point titration technique [14,15]. In addition we have directly measured the $\Delta\bar{\mu}H^+$ generated by ATP hydrolysis in both systems using ion distribution techniques. We find that from a comparison of these two values, that the H^+/ATP ratio in intact mitochondria is close to 3 whereas in phosphorylating sub-mitochondrial particles it is approximately 2.

Materials and Methods

Mitochondria from etiolated mung bean hypocotyls (*Phaseolus aureus*) were prepared according to the general method described by Bonner [35]. Sub-mitochondrial particles were prepared by careful dilution of the mitochondria to 35 mosM with cold distilled water containing 1 mM ATP, 1 mM $MgCl_2$, 15 μM bovine serum albumin, and 10 mM Hepes (pH 7.2), followed by French pressing at 3000 lbs/inch^2. Following centrifugation at 10 000 × *g* for 10 min to remove unbroken mitochondria, particles were sedimented at 108 000 × *g* for 40 min. Particles prepared in this manner rapidly oxidized NADH and succinate as respiratory substrates. Respiration was stimulated by uncoupling agents.

Respiration rates were monitored with a Rank oxygen electrode (Rank Bros. Bottisham, Cambridge) in a closed chamber at 25°C in 2 ml of reaction medium containing 0.3 M mannitol, 10 mM KCl, 5 mM $MgCl_2$ and 10 mM potassium phosphate buffer (pH 7.2).

The steady state of protonmotive force ($\Delta\bar{\mu}H^+$) in mitochondria was measured following silicone oil centrifugation by an ion distribution method as previously described [11] using $^{86}RbCl$ (0.1 μCi/ml) as a membrane potential ($\Delta\psi$) marker and sodium [^{14}C]acetate (0.3 μCi/ml) to estimate the ΔpH gradient in 1 ml of medium containing 0.3 M mannitol, 5 mM $MgCl_2$, 20 mM Hepes buffer (pH 7.2) and 10 ng/ml valinomycin and approx. 3 mg mitochondrial protein.

KS[^{14}C]N (0.2 μCi/ml) and [^{14}C]methylamine hydrochloride (0.3 μCi/ml) were used in parallel experiments, to determine $\Delta\psi$ and ΔpH, respectively, in sub-mitochondrial particles in a medium containing 0.2 M mannitol, 50 mM KCl, 5 mM $MgCl_2$, 20 mM Hepes buffer (pH 7.2) and 3—5 mg protein. Following incubation the particles were rapidly sedimented using a Beckman air-fuge for 1 min at 110 000 $\times g$. After centrifugation the pellet was rapidly removed from the supernatant, wiped to remove residual water from the walls of the tube, and placed in 14% perchloric acid. In both cases 100 μl samples were counted in 5 ml of Handifluor cocktail mixture (Mallikrodt Chemical Co.) in an intertechnique liquid scintillation spectrometer. The sucrose permeable and impermeable spaces in non-energized mitochondria and particles were determined in parallel experiments using [^{14}C]sucrose (1 μCi/ml) and ^3H_2O (10 μCi/ml). The matrix space was found to be approx. 1.9 μl/mg and 1.2 μl/mg for mitochondria and sub-mitochondrial particles, respectively. $\Delta\bar{\mu}H^+$ was then derived from the arithmetic sum of $\Delta\psi$ and ΔpH.

Colored extrinsic probes were used as indicators of the membrane potential in both mitochondria and particles and were used to determine the steady state phosphorylation potential (ΔG_p) using the null point technique developed by Bashford and Thayer [15]. Media employed to monitor absorbance changes in mitochondria and particles were identical to that used to measure $\Delta\bar{\mu}H^+$ without the addition of valinomycin and the radioactively labelled ions. The valinomycin concentration used in the ion distribution experiments was chosen such that it caused no detectable changes in probe response, suggesting that at a concentration of 10 ng/ml it does not reduce $\Delta\psi$ significantly. A Johnson Research Foundation double bean spectrophotometer was used to monitor changes in absorbance at the wavelengths specified in the figure legends. Safranine (16 μM or approx. 25 nmol/mg protein) was used to monitor membrane potential in mitochondria [16] whereas oxonol-VI (approx. 5 nmol/mg protein) was used with submitochondrial particles [15]. A standard fluorimetric procedure was used to estimate the concentrations of ATP and ADP enzymatically as described in Ref. 36. Inorganic phosphate was determined according to the method of Fiske and SubbaRow [37].

Protein was determined by the method of Lowry et al. [38] using crystalline bovine serum albumin as a standard.

Valinomycin and safranine O were purchased from Sigma Chemical Co. and dissolved in ethanol and water, respectively. The dye OX-VI (dissolved in absolute ethanol) was a gift from Dr. J.C. Smith (Johnson Research Founda-

tion). All isotopes were obtained from New England Nuclear (Boston, MA). All other reagents were of the highest purity commercially available.

Results

The energy-linked responses of both safranine and OX-VI are shown in Fig. 1. The initiation of respiration in mitochondria upon addition of 1 mM NADH resulted in an increase in absorbance of the safranine corresponding to the establishment of a membrane potential (Fig. 1A). At the dye/protein ratio used in this study (namely 25 nmol/mg protein) the time response of the probe was very rapid and resulted in much larger absorbance changes than observed by Akerman and Wikström [6]. At this ratio, however, safranine absorbance changes were found to be non-linear with respect to a valinomycin-induced K^+ diffusion potential at membrane potentials above 170 mV. This non-linear response may be indicative of a significant amount of safranine binding [39] or alternatively may be due to an underestimation of extra mitochondrial potassium since this was estimated as added potassium [6]. Nevertheless, the sensitivity of the probe at this constant dye/protein ratio rendered it a suitable indicator for monitoring changes in mitochondrial membrane potential. NADH was used as a respiratory substrate since, in plant mitochondria, it is oxidized on the outer surface of the inner membrane [40] and therefore does not require translocation into the matrix. Furthermore, the well-known phenomena of 'conditioning' [41], a situation in which the initial rate 3 is not as rapid as

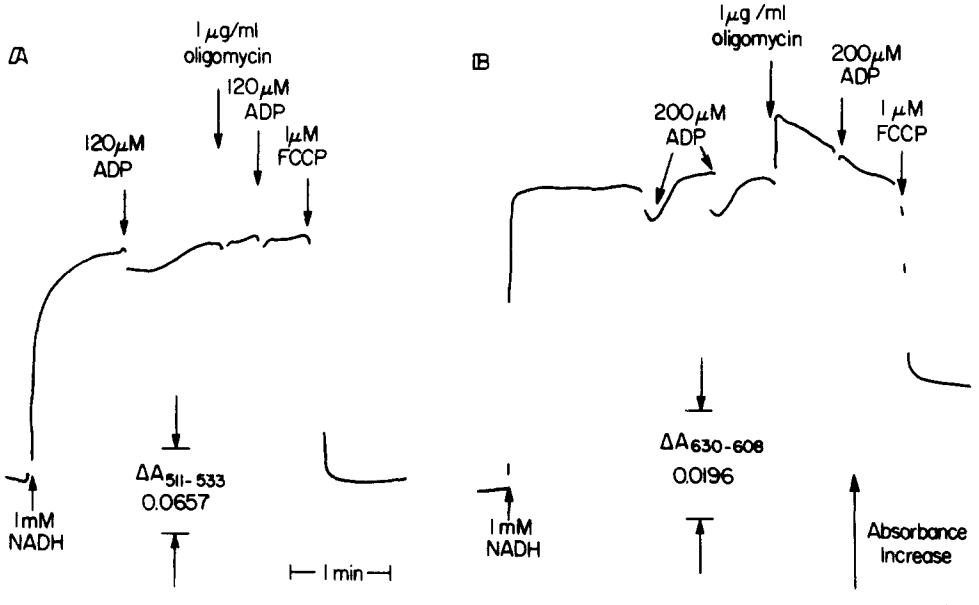

Fig. 1. Energy linked responses of safranine and OX-VI. In A, approx. 0.62 mg/ml of mitochondrial protein was incubated in 2.5 ml of medium containing 0.3 M mannitol, 5 mM $MgCl_2$, 20 mM Hepes buffer (pH 7.2), 1 mM sodium phosphate and 16 μM safranine. In B, approx. 0.83 mg/ml of sub-mitochondrial particles was incubated in 2.5 ml of medium containing 0.2 M mannitol, 5 mM $MgCl_2$, 50 mM KCl 20 mM Hepes buffer (pH 7.2) and 1.6 μM OX-VI. Absorbance changes were monitored at the wavelengths indicated. Respiration was initiated by the addition of 1 mM NADH.

subsequent state 3 rates, is not normally observed with NADH. However, a similar magnitude of shift was obtained irrespective of whether the substrate was NADH, succinate, or malate. The addition of 20 ng/ml nigericin (not shown) resulted in an increase in absorbance suggesting that the dye had the capability of responding to further increases in membrane potential. It can be seen from Fig. 1A that upon addition of limiting amounts of ADP during NADH respiration, there is a rapid decrease in membrane potential which returns to its initial value upon subsequent transition to state 4. Such cyclic responses were abolished by 1 μg/ml oligomycin. Similarly 1 μM FCCP or anaerobiosis collapsed any absorbance changes. These results are in agreement with data obtained using the Rb^+ distribution technique [42].

Fig. 1B shows similar experiments in which the oxonol dye, OX-VI, was used to monitor the membrane potential in phosphorylating sub-mitochondrial particles. The sub-mitochondrial particles used in this investigation were approx. 76% oriented as measured by exogenous cytochrome *c* stimulation of NADH oxidation and inhibition by 0.25 μM poly(L)lysine [43]. Addition of 1 μM FCCP stimulated NADH oxidation approx. 3- to 4-fold. NADH respiration caused a rapid absorbance increase at 630 nm, which remained relatively stable until anaerobiosis. The addition of oligomycin resulted in a transient increase in signal which subsequently declined to its initial value. Oligomycin was also found to induce an increase in respiratory coupling under these conditions suggesting that it possibly improves $\Delta\psi$ by blocking proton leakage. As observed in Fig. 1A, no cyclic responses to ADP were observed following oligomycin addition. It may be noted from Fig. 1B, that in agreement with Sorgato et al. [21] 1 μM FCCP did not totally collapse the oxonol response. This only occurred upon anaerobiosis or in the presence of a respiratory inhibitor. Again ATP hydrolysis caused a probe response of similar magnitude. A dye/protein ratio of 2 nmol/mg was used in the experiment shown in Fig. 1B and although at this ratio the extent of the probe response was large, in subsequent experiments a protein concentration of 0.3 mg/ml was employed in order to ensure that the signal was not saturated (see Ref. 15) and could respond to both increases and decreases in membrane potential. 50 mM potassium chloride was included in the reaction medium, since it was found that the response of the probe in this medium was much more stable than in media of low ionic strength and there was relatively little decline in its response with time. Under these conditions, 20 ng/ml nigericin increased the extent of the probe response approx. 40% suggesting the presence of a significant pH gradient in respiring particles suspended in mannitol/KCl medium.

Presumably, the pH gradient is due to the presence of Cl^- in the incubation medium acting as an electrically permeant ion.

In this study, however, we have only monitored the electrical component of the electrochemical proton gradient since pH indicators such as neutral red responded too slowly, under our conditions, to pH changes induced by ADP and FCCP additions. Furthermore, facilities were not available to monitor fluorescent changes of the probe, 9-aminoacridine. It should also be stressed, that at the concentrations used in this study, both dyes in contrast to cyanide dyes [39,44,45] had no deleterious effect on the coupled NADH respiration of either mitochondria or sub-mitochondrial particles.

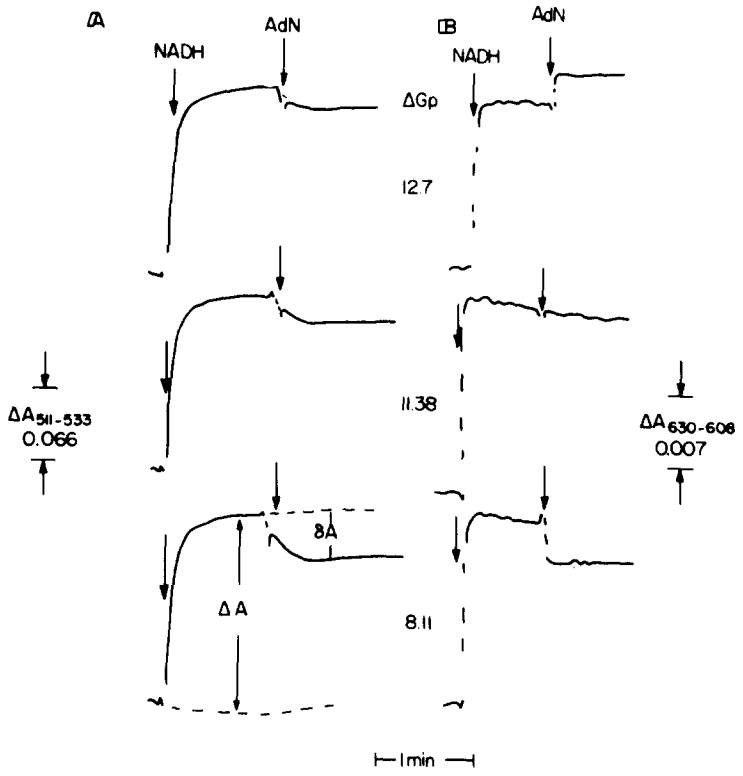

Fig. 2. The effect of phosphate potential on the energy-linked response of safranine (A) and OX-VI (B) during NADH respiration. The extent of the spectral change (δA) relative to the steady-state level of absorbance (ΔA) were recorded following addition of ATP and ADP mixtures (1.0 mM final concentration) at ATP/ADP ratios between 0.01 and 10, and P_i concentrations between 2 and 5 mM. Absorbance changes were measured as described for Fig. 1. The imposed ΔG_p was calculated from the relationship: $\Delta G_p = -\Delta G'_0 + RT \ln[(ATP]/[ADP][P_i])$ where $\Delta G'_0$ is the standard free energy of ATP hydrolysis. Fig. 2A shows the spectral changes observed using mitochondria with safranine (25 nmol/mg protein) whereas in 2B, sub-mitochondrial particles with OX-VI (5 nmol/mg protein) were used. Respiration was initiated by the addition of 1 mM NADH.

The extent of the absorbance changes observed upon addition of ADP (with both safranine (Fig. 1A) or OX-VI (Fig. 1B), during steady-state NADH respiration, was not dependent upon the concentration of ADP but on the ratio of [ATP]/[ADP][P_i] (i.e. the phosphorylation potential). This effect was further investigated by varying the ATP/ADP ratio (between 0.01 to 10) at constant P_i (5 mM) concentrations or by varying the P_i concentration (0.1 to 9 mM) at a constant ATP/ADP ratio (10 : 1) (Fig. 2). The adenine nucleotides were added by a single addition to give a final concentration of 1 mM. The responses were stable for at least 1 min following addition of the adenine nucleotides. A $\Delta G'_0$ value of −7.3 kcal/mol for ATP hydrolysis [17] was used to calculate the value of a ΔG_p whilst the concentration of ATP, ADP and P_i were determined.

In submitochondrial particles (Fig. 2B), high ΔG_p values (above 11 kcal/mol) resulted in an increase in the extent of the probe response whereas below a ΔG_p of 11.4 kcal/mol, a decrease in absorbance was observed, upon addition of the adenine nucleotide mixtures. No increase in signal size was observed in the

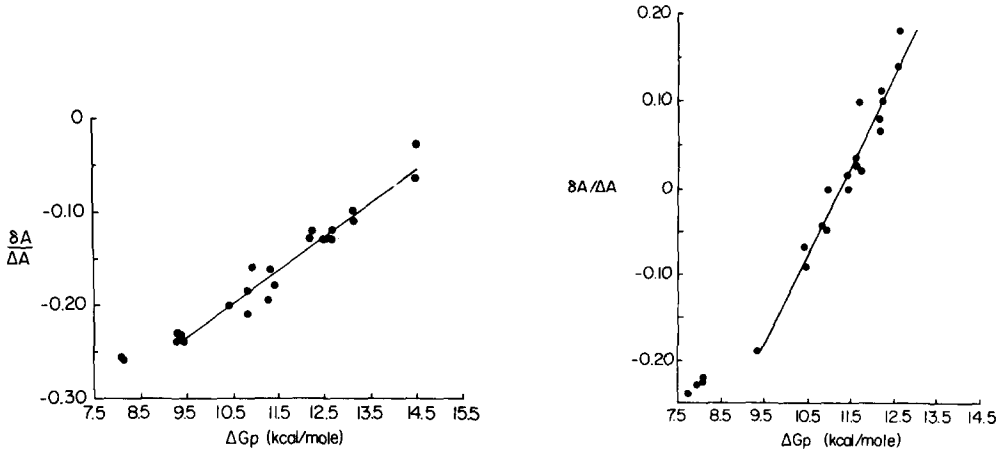

Fig. 3. The effect of ΔG_p on the extent of the spectral responses of safranine during NADH respiration. The fractional absorbance change $\delta A/\Delta A$ (see Fig. 2) was plotted against the phosphate potential imposed by the addition of the adenine nucleotide mixtures. The relationship was obtained by linear regression, where $r = 0.93$. ΔG_p was varied by the addition of various adenine nucleotide mixtures at ATP/ADP ratios between 0.01 and 10, and P_i concentrations between 0.1 and 9 mM. Experiments were performed as indicated in Fig. 2.

Fig. 4. The effect of ΔG_p on the extent of the spectral response of OX-VI during NADH respiration. $\delta A/\Delta A$ (see Fig. 2) was plotted against ΔG_p as in Fig. 3. ΔG_p was varied as in Fig. 3. The line was fitted by linear regression where $r = 0.94$.

mitochondrial experiments at the ΔG_p values used in this study. In all cases, a reduction of the absorbance change occurred, the extent of which was related to the ΔG_p imposed by the adenine nucleotide mixtures. In the mitochondrial experiments a second aliquot of adenine nucleotides was made after the potential had been collapsed by valinomycin, oligomycin and FCCP in order to account for any absorbance change due to binding of the ATP with the safranine. Typical corrections were in the region of 0.003 δA. No such corrections were required with OX-VI (see Ref. 15). A typical plot of the fractional absorbance change ($\delta A/\Delta A$) against the imposed phosphate potential for mitochondria and sub-mitochondrial particles is shown in Figs. 3 and 4, respectively. In each case, the line was fitted by linear regression where $r = 0.93$ (Fig. 3) and 0.94 (Fig. 4). From an analysis of the probe response as a function of ΔG_p (Figs. 3 and 4) it can be seen that in both cases that the extent of the absorbance change of a wide [ATP]/[ADP][P_i] concentration (ΔG_p values between 9.5 and 13.5 kcal/mol) is linearly dependent on ΔG_p. Deviation from linearity was observed only at very high and low phosphorylation potentials. Extrapolation of the measured dependence of the safranine response (Fig. 3) on the imposed ΔG_p, indicates that the nullpoint phosphorylation potential occurred at 15.2 ± 0.7 kcal/mol (from five independent measurements). With submitochondrial particles, the ΔG_p which caused no change in the optical signal of OX-VI was 11.2 ± 0.3 kcal/mol (from nine independent experiments). In the presence of 0.25 μM poly(L-lysine) the 'nullpoint' ΔG_p corresponded to 11.5 kcal/mol for OX-VI. Under these conditions mitochondrial NADH oxidation (uncoupled) was inhibited 90% whereas respiration in particles was inhibited

22%. Since poly(L-lysine) inhibits the oxidation of exogenous cytochrome *c* [43] this result indicates that the particles used in this study are composed of a mixed population of inside out and right side out vesicles.

Nevertheless, it is readily apparent that the extramitochondrial ΔG_p maintained during respiration by intact mitochondria is approx. 4 kcal/mol higher than that observed with coupled sub-mitochondrial particles.

The magnitude of the electrochemical proton gradient generated by the hydrolysis of ATP was measured directly using ion distribution techniques. Mitochondria and sub-mitochondrial particles were incubated for 5 min in order to establish a maximal potential before centrifugation. These experiments were performed in media identical to that used in the ΔG_p experiments with mitochondria or particles from the same preparation. $\Delta\psi$ (in mitochondria) was estimated from a distribution of $^{86}Rb^+$ whereas S[^{14}C]N$^-$ was used with particles. Parallel experiments were used to determine ΔpH in particles using [^{14}C]methylamine hydrochloride. From three independent experiments, the sucrose impermeable space was found to be 1.9 μl/mg and 1.2 μl/mg for mitochondria and sub-mitochondrial particles, respectively. The components of $\Delta\bar{\mu}H^+$ maintained in either case are summarized in Table I. In both cases, the hydrolysis of ATP generated both a membrane potential of 90—100 mV and a substantial pH gradient. From four separate experiments a value of 193 ± 10 mV was obtained for $\Delta\bar{\mu}H^+$ in mitochondria, whilst ATP hydrolysis in submitochondrial particles maintained a $\Delta\bar{\mu}H^+$ of 224 ± 12 mV (three experiments). The pH gradient in the particles was found to be appreciably larger than that observed with mitochondria. The addition of 1 μg/ml oligomycin or 1 μM FCCP under these conditions partially collapsed both components (not shown, see Ref. 41). It may be concluded from Table I, that submitochondrial particles are able to maintain a protonmotive force of similar magnitude to that found in mitochondria.

If the 'null point' ΔG_p values obtained here are converted into electrical units and are compared to the steady state protonmotive force an estimate of the number of protons translocated across the membrane per ATP synthesized

TABLE I

AN ESTIMATION OF $\Delta\bar{\mu}H^+$ GENERATED BY THE HYDROLYSIS OF ATP

Approx. 3 mg mitochondrial protein was incubated for 5 min in 1 ml of a medium containing 0.3 M mannitol, 5 mM $MgCl_2$, 20 mM Hepes (pH 7.2, with Tris-HCl), 10 ng/ml valinomycin, 10 μCi/ml ^3H_2O, 0.3 μCi/ml sodium [^{14}C]acetate, 0.01 μCi/ml ^{86}RbCl and 1 mM ATP. Following incubation, the mitochondria were rapidly separated from the external medium by centrifugation through silicone oil and analyzed. With submitochondrial particles, approx. 3—5 mg protein was incubated for 5 min in 150 μl of medium containing 0.2 M mannitol, 50 mM KCl, 5 mM $MgCl_2$, 20 mM Hepes (pH 7.2), 10 μCi/ml ^3H_2O, 0.2 μCi/ml KS[^{14}C]N or 0.3 μCi/ml [^{14}C]methylamine and 1 mM ATP. The $\Delta\psi$ and ΔpH components were estimated from parallel experiments. Following incubation, the particles were rapidly separated in an Air-fuge for 1 min and markers analyzed. The values are a mean and standard deviation of four (mitochondria) and three (particles) determinations.

	$\Delta\psi$ (mV)	ΔpH	$-Z\Delta$pH (mV)	$\Delta\bar{\mu}H^+$ (mV)
Mitochondria	92 ± 10	1.69 ± 0.17	101 ± 10	193 ± 10
Submitochondrial particles	97 ± 8	2.12 ± 0.20	127 ± 12	224 ± 12

TABLE II

A COMPARISON OF $\Delta\bar{\mu}H^+$ WITH THE PHOSPHATE POTENTIAL

Data used in this table was taken from the null-point ΔG_p values and Table I. The conversion of ΔG_p into electrical units was performed according to the relationship ΔG_p (mV) = ΔG_p (kcal/mol)/F where F is the Faraday constant (F = 23.063 kcal/V equivalent). The apparent H^+/ATP ratio was calculated from the phosphate potential (mV) divided by $\Delta\bar{\mu}H^+$ (mV).

	$\Delta\bar{\mu}H^+$ (mV)	ΔG_p		Apparent H^+/ATP
		kcal/mol	mV	
Mitochondria	193 ± 10	15.2 ± 0.7	659 ± 30	3.4 ± 0.2
Submitochondrial particles	224 ± 12	11.2 ± 0.3	485 ± 13	2.2 ± 0.1

can be made (Table II). The data presented in Table II indicates that the apparent H^+/ATP ratio has a value of 3.4 ± 0.2 in mitochondria and 2.2 ± 0.1 in submitochondrial particles.

Discussion

The purpose of this study was to determine the phosphorylation potential normally maintained by respiring plant mitochondria and sub-mitochondrial particles and to compare this with a direct measurement of the magnitude of the electrochemical proton gradient in order to ascertain the stoichiometry of proton transport by the ATPase. Extrinsic probes were used to monitor the membrane potential in both respiring mitochondria and sub-mitochondrial particles and have been used to determine the phosphorylation potential normally maintained under these conditions. The use of probes as a measure of the prevalent ΔG_p has the advantage over other techniques in so much as the experimental procedures are simple and continuous, and furthermore do not perturb the system [46] (For example centrifugation techniques may result in a change in the phosphorylation potential during centrifugation). The difficulty of accurate calibration of probe response (i.e. Ref. 39) is not encountered in this study, since only the condition which resulted in no change in optical signal upon addition of adenine nucleotide mixtures were determined.

The effects of ADP, oligomycin and FCCP on the energy-linked responses of safranine and OX-VI on plant mitochondria and sub-mitochondrial particles are in agreement with previous studies [15,46] that these probes monitor membrane potential changes in energy-conserving systems. The present experiments suggest that a large membrane potential is maintained during steady-state respiration in both mitochondria and particles, which declines slightly under state 3 conditions, consistent with the idea that ATP synthesis puts an energetic demand on the protonmotive force. Furthermore, the large energy-linked shifts of OX-VI suggest that the sub-mitochondrial particles used in this study are reasonably well coupled and are capable of maintaining a membrane potential during steady-state respiration. The addition of adenine nucleotide mixtures, as previously observed [14,15], altered the membrane potential in both respiring mitochondria and sub-mitochondrial particles, with both probes having an approximately linear dependence on the imposed ΔG_p.

In this study, the optical probe techniques indicated that the ΔG_p observed with intact mitochondria has a value of 15.2 ± 0.7 kcal/mol whereas in sub-mitochondrial particles it has a value of 11.2 ± 0.3 kcal/mol. These latter values are comparable to the recently reported values of 10.3 to 11.9 kcal/mol for the intra-mitochondrial ΔG_p observed by null point and other techniques [15,21], again indicative of a well coupled system. It is of interest to note that a titration of the safranine response gave a ΔG_p value, upon extrapolation, very close to that observed in suspensions of intact mitochondria using totally different techniques [17—20]. The null point measurements reported here are also further confirmation of the observation [14,17—21], that respiring mitochondria maintain an external phosphorylation potential that is approximately 4 kcal/mol higher than its internal counterpart. It is, of course, conceivable that since the ΔG_p determinations are based only on the $\Delta\psi$ null point, even though the major contribution to $\Delta\bar{\mu}H^+$ in particles is ΔpH, the addition of adenine nucleotides (an electrogenic process) merely induces H^+ redistributions that change the contribution of either component. We have noted, however, that the inclusion of nigericin gave a null point ΔG_p comparable to that observed in its absence and furthermore it is apparent from the data of Bashford and Thayer [15] that the $\Delta\psi$ and ΔpH null points are very similar. With respect to the magnitude of the ΔG_p maintained by respiring particles, it is possible that its value is higher than that measured in this report. An artifically low null point may well be observed merely due to contamination by a certain amount of uncoupled particles, for it is obvious that the particles used in these experiments are not homogenous. Presumably partly uncoupled particles would tend to show an increase in oxonal absorption at ΔG_p where fully coupled particles would still show a decrease, thus causing a relative underestimation of the null point ΔG_p. Cross-contamination by right-side out particles is, of course, reduced in our experiments by the inclusion of poly(L-lysine). That the lower ΔG_p is not merely a reflection of the poor coupling properties of the particles is indicated by the magnitude of the electrochemical protein gradient generated upon ATP hydrolysis. The presence of right side and uncoupled particles would not, of course, contribute much to the $\Delta\bar{\mu}H^+$ since neither of these would accumulate methylamine or SCN^-. The method of measuring $\Delta\bar{\mu}H^+$ is, however, heavily in favor of particles that maintin the highest $\Delta\bar{\mu}H^+$. Nevertheless it is evident, from Table I, that particles are able to maintain a protonmotive force of similar magnitude to that found in mitochondria and which has a value of approx. 200—220 mV. In contrast to recent reports [21,34] however this is composed of both $\Delta\psi$ and ΔpH components, the latter being the larger. Furthermore these measurements are consistent with the notion [15] that, in high salt media, $\Delta\psi$ has a value of between 90 to 100 mV. Previous measurements by Sorgato et al. [21] and Ferguson et al. [34] on the magnitude of $\Delta\bar{\mu}H^+$ normally maintained in respiring sub-mitochondrial particles are considerably lower than the values reported here, although in a recent communication Berry and Hinkle [47] have measured values comparable to this report. The values obtained in Ref. 21 may possibly be due to the lack of any detectable pH gradient for it is apparent from Table I that this composes approximately 57% of the total $\Delta\bar{\mu}H^+$ in sub-mitochondrial particles. Although it must be stressed that the two components are interconvertible and therefore dependent

on media composition.

From a comparison of the protonmotive force with the value of the phosphorylation potential obtained using the null point technique, a value of 3.4 and 2.2 is obtained for the H^+/ATP ratio in mitochondria and sub-mitochondrial particles. The reason why the ratio is not an integral is unclear, however, it could merely be a reflection of several sources of error. In our experiments the most likely sources of error, are due either to an underestimation of the protonmotive force or an overestimation of ΔG_p. An underestimation of the protonmotive force may arise from an inaccurate determination of the sucrose impermeable space. A value of 1.2 μl/mg measured in this study is considerably higher than that used by other workers [10], and may possibly reflect mitochondrial contamination. The result that the particles used in this study consist of a mixed population of inside-out and right-side vesicles would tend to support this idea. Since the protonmotive force measured in this study, is however much higher than previously reported (see Ref. 21), the error in overestimating the sucrose space is probably minimal. An error in the estimation of the phosphorylation potential is likely, particularly with respect to mitochondria. For in this case a null point is obtained only upon extrapolation whilst in sub-mitochondrial particles, it is clearly seen. An error in either one of these values could explain a non-integral number for the H^+/ATP ratio. Nevertheless, it is apparent that the ΔG_{ATP} is approximately twice $\Delta\bar{\mu}H^+$ in sub-mitochondrial particles and three times $\Delta\bar{\mu}H^+$ in mitochondria, consistent with the viewpoint that in intact mitochondria an extra proton is required for the uptake of P_i and the electrogenic transport of ATP^{4-} outwards in exchange for internal ADP^{3-}. Indeed direct determinations of the H^+/ATP ratio in submitochondrial particles [28] and in mitochondria [22,26] have also indicated a ratio of 2 for the ATPase (however see Ref. 48).

Such stoichiometries can be accomodated either by a mitochondrial P/O ratio of 2 for NADH oxidation [33] or alternatively a H^+/O ratio of 9 or greater [49]. The alternative interpretation (see Ref. 50) is that there are certain theoretical reasons why the stoichiometry should not be an integral number, but rather be lower. In which case it could be argued that the H^+/ATP ratios for sub-mitochondrial particles and mitochondria are 3 and 4, respectively, which would be more in accordance with Refs. 25 and 34.

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