Two protons are pumped from the mitochondrial matrix per electron transferred between NADH and ubiquinone

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The spectrophotometric indicators neutral red and safranine were used to determine the relative H^+/e^- ratios of proton uptake from the mitochondrial matrix, and the q^+/e^- ratios of electrical charge translocation, during oxidation of β-OH-butyrate and succinate by ferricyanide in rat liver mitochondria. With β-OH-butyrate both ratios were higher than with succinate by a factor close to 3.0. Since there is full agreement that H^+/e^- of proton uptake and q^+/e^- of charge translocation are both equal to unity for oxidation of succinate (or ubiquinol) by ferricytochrome *c*, the corresponding ratios for oxidation of NADH by ubiquinone and cytochrome *c* are near 2.0 and 3.0, respectively.

H^+ translocation *H^+/e^- stoichiometry* *Mitochondria* *Energy conservation*

1. INTRODUCTION

The only respiratory chain segment for which there now is full agreement on the H^+/e^- stoichiometry of redox-linked proton translocation is ubiquinol-ferricytochrome *c* oxidoreductase. Here, 1 H^+ is taken up from the mitochondrial matrix and 2 H^+ are released on the cytoplasmic or C side of the membrane for each transferred electron. Of the latter, 1 H^+ stems from ubiquinol and is released non-electrogenically. Hence, there is translocation of one electrical charge equivalent per electron transferred from ubiquinol to ferricytochrome *c* (see [1,2]).

In contrast, the corresponding H^+/e^- and q^+/e^- stoichiometries for the segments between NADH and ubiquinone ('site 1'), and cytochrome *c* and O_2 ('site 3') have long remained controversial. This is unfortunate because knowledge of the stoichiometric aspect of these reactions is a prerequisite for elucidation of their mechanism, and for any quantitative thermodynamic analysis. In the past, mitochondrial proton translocation has been measured mainly by determination of H^+ ejection from mitochondria. In only relatively few instances have there been attempts to determine the q^+/e^- ratio. Particularly the former but also the latter measurements are beset with technical difficulties which may partly explain these discrepancies (see [2–4]).

To exclude artefacts and to prove transport one must ultimately determine the appearance and disappearance of the transported species quantitatively on the two sides of the membrane. For mitochondrial proton transport, attempts at the latter have so far been restricted to measurements of proton uptake into inverted inner mitochondrial membrane vesicles (so-called submitochondrial particles). However, their heterogeneity, poor coupling characteristics and small internal volume make such measurements uncertain. It would therefore be important to measure quantitatively proton uptake from the mitochondrial matrix.

Authors in [5,6] have shown that the pH-indicator neutral red (NR) penetrates biological membranes, and that it may be used to measure qualitatively changes in intravesicular pH in suspensions of chloroplasts containing high concentrations of impermeant buffer. Similar results have been re-

Abbreviations: NEM, *N*-ethylmaleimide; nig, nigericin; NR, neutral red; P_i, inorganic phosphate; Q, ubiquinone; QH_2, ubiquinol; q^+/e^-, ratio of translocation of electrical charge equivalents per transferred electron; val, valinomycin

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ported for mitochondria [7,8]. Recently, I reported that the NR signal may be successfully calibrated in terms of ΔH^+ in the mitochondrial matrix by making use of the known uptake of $1\,H^+/e^-$ during oxidation of ubiquinol (or succinate) by ferricytochrome *c* [9]. With this technique it was demonstrated that there is uptake of $2\,H^+/e^-$ from the matrix during oxidation of ferricytochrome *c* by O_2, as catalysed by cytochrome oxidase [9]. This agrees quantitatively with the proton pump function of the oxidase proposed previously [1–3,8]. Here this technique is applied to determine the H^+/e^- ratio of proton uptake linked to oxidation of NADH by ubiquinone. In addition, a new technique is introduced to determine the q^+/e^- ratio using safranine (see [10]) as a probe of charge displacement.

2. MATERIALS AND METHODS

Rat liver mitochondria were isolated in a 0.25 M sucrose–0.1 mM EGTA medium, as in [11]. They were suspended at a concentration of 10 μM cytochrome aa_3 (approx. 71 mg protein/ml) in the same medium without EGTA, and kept on an ice-water bath. The content of cytochrome aa_3 was determined at 605–630 nm by reduction with dithionite, using an extinction coefficient of $27\,mM^{-1}\cdot cm^{-1}$.

The basic reaction mixture contained 100 mM sucrose, 20 mM KCl and 50 mM Hepes, pH 7.4 (adjusted with KOH). The final concentration of mitochondria corresponded to 0.83 μM cytochrome aa_3 (about 5.9 mg protein/ml). Further additions are specified in the figure legends. A DBS-1 dual-wavelength spectrophotometer designed and constructed at the Johnson Research Foundation workshops (University of Pennsylvania) was used in all experiments. The cuvettes had a 1 cm light path and were thermostatted at 25.0°C.

All experiments were conducted by adding small calibrated aliquots of potassium ferricyanide to initiate brief respiratory bursts, which lasted only for a few seconds. This elicited relatively fast ($t_{1/2} < 5$ s) absorption changes of NR or safranine, which after consumption of the added oxidant returned to the baseline, usually with a $t_{1/2}$ of approx. 40–60 s. This technique is analogous to the oxidant-pulse method in [12], where ejection of H^+ or uptake of K^+ is measured extramitochondrially with ion-selective electrodes. The decays of the absorption signals were extrapolated to a point half-way between addition and exhaustion of the oxidant (cf. [12,13]), from which the extent of absorption change was obtained. This procedure is permissible due to the much faster velocity of the oxidant-induced absorption change, in comparison with its subsequent decay (see fig.1 and [12,13]).

3. RESULTS AND DISCUSSION

Fig.1 shows the matrix alkalinisation as measured by NR after addition of a small amount of ferricyanide in the presence of rotenone, cyanide and valinomycin (cf. section 2). The net alkalinisation is greatly diminished if NEM is absent, and is further decreased in such conditions on addition of potassium phosphate (not shown). If the valinomycin is left out the absorption change is also diminished, and if EGTA is also present to chelate Ca^{2+}, there is virtually no response at all (not shown). In the presence of nigericin (or uncoupling agents) the relaxation of the alkalinisation pulse is greatly accelerated. At higher concentrations of either nigericin or uncoupling agents, there is virtually no net alkalinisation after addition of the oxidant. All these findings are consistent with the

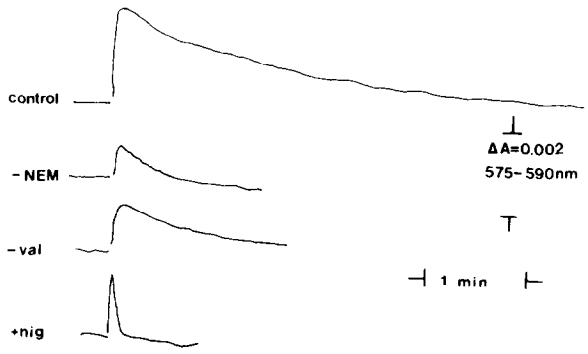

Fig.1. Intramitochondrial alkalinisation linked to oxidation of succinate by ferricyanide. The basic reaction mixture (see section 2) was supplemented with 3 μM rotenone, 1.3 mM KCN, 0.21 μg/ml valinomycin (val), 4.2 mM potassium succinate, 83 μM neutral red, 0.21 mM *N*-ethylmaleimide (NEM) and rat liver mitochondria equivalent to 0.83 μM cytochrome aa_3. Final volume 1.2 ml. The reaction was initiated by addition of 33 μM potassium ferricyanide. In addition to the control, experiments are shown in which either the NEM or the valinomycin was absent, or where 83 ng/ml of nigericin (nig) was present additionally.

notion that NR measures intramitochondrial alkalinisation caused by redox-linked proton translocation. Very similar observations were made in the absence of rotenone and with β-OH-butyrate replacing succinate (cf. below).

Fig.2 shows that the extent of absorption of NR is linearly related to the amount of oxidant added in the measured range both with succinate (cf. [9]) and β-OH-butyrate as substrate. However, the slope of the plot is 3-times steeper with β-OH-butyrate than with succinate (plus rotenone). In 14 experiments with 6 different batches of mitochondria the mean factor observed was 2.82 (±0.13 SD). Hence, 3-times more protons are taken up from the matrix phase per transferred electron when β-OH-butyrate is oxidised, as compared with oxidation of succinate. Since the oxidation of succinate (or ubiquinol) by ferricytochrome *c* is known to be accompanied by uptake of 1 H^+/e^- (see [1,2]), this means that electron transfer between NADH and ubiquinone ('site 1') is linked to uptake of 2 H^+/e^-.

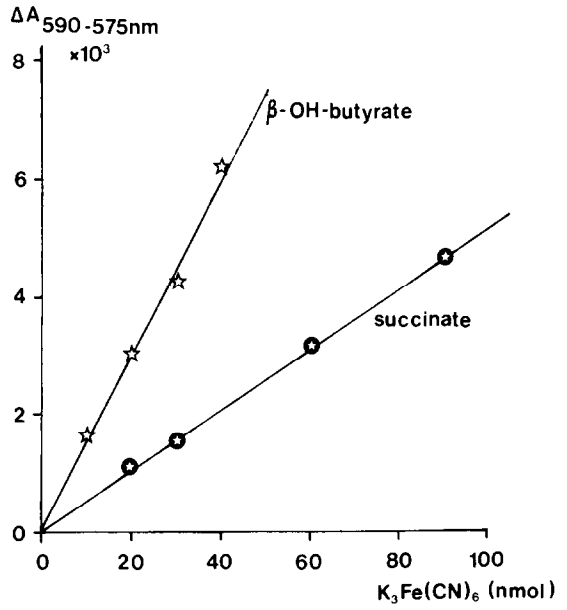

Fig.2. Extent of intramitochondrial alkalinisation with succinate and β-OH-butyrate as substrates. The conditions were those described in the legend to fig.1, except that in the β-OH-butyrate experiments rotenone was absent and 8.3 mM potassium β-OH-butyrate replaced the succinate. The abscissa refers to different amounts of potassium ferricyanide added in different experiments.

Authors in [10] introduced safranine as a probe of the mitochondrial membrane potential. The mechanism by which this probe measures charge displacement is probably an electric field-induced accumulation of the positively charged safranine molecules on the internal surface of the membrane, which results in their 'stacking' [14] and quenching of light absorption.

Similar experiments to those reported in fig.1 (and see section 2) were conducted in the presence of safranine in place of NR. Valinomycin, which together with K^+ abolishes the safranine response ([8]; constrast the NR response), was not present in these experiments.

Fig.3 shows the extents of safranine absorption changes plotted against the amount of added oxidant. With the smallest amounts of ferricyanide the absorption increases more slowly than with higher amounts when the response becomes linear. Extrapolation of the linear portions to the abscissa yields the amount of oxidant that apparently causes no change in safranine absorption. The initial curvature is presumably due to movement of charged molecules other than safranine in response to the field, such as endogenous Ca^{2+}, so that charging of the membrane (as measured by safranine stacking) ensues only after saturation of this electric field-induced ion movement and/or dipole alignment. The subsequent linear increase in absorption change has a slope that is 3-times greater with β-OH-butyrate than with succinate as substrate. The mean of 9 experiments with 4 different mitochondrial batches was 2.80 (±0.09 SD). Also the intercepts with the abscissa suggest that β-OH-butyrate is 3-times more effective than succinate in charging the membrane, per transferred electron. Here the mean of the ratio between intercepts with β-OH-butyrate and succinate was 2.68 (±0.10 SD; $N = 9$). Since the q^+/e^- ratio is agreed to be unity for oxidation of succinate (or ubiquinol) by cytochrome *c* (site 2; see [1,2]), these results indicate that the corresponding ratio is 3 for the span between NADH and cytochrome *c*, and 2 for the span between NADH and ubiquinone.

These measurements of proton uptake from the mitochondrial matrix, and of electrical charge displacement, strongly support the suggestion in [15,16] that the H^+/e^- and q^+/e^- ratio is 2 for the site 1 region of the respiratory chain, between NADH and ubiquinone. The energy-conserving

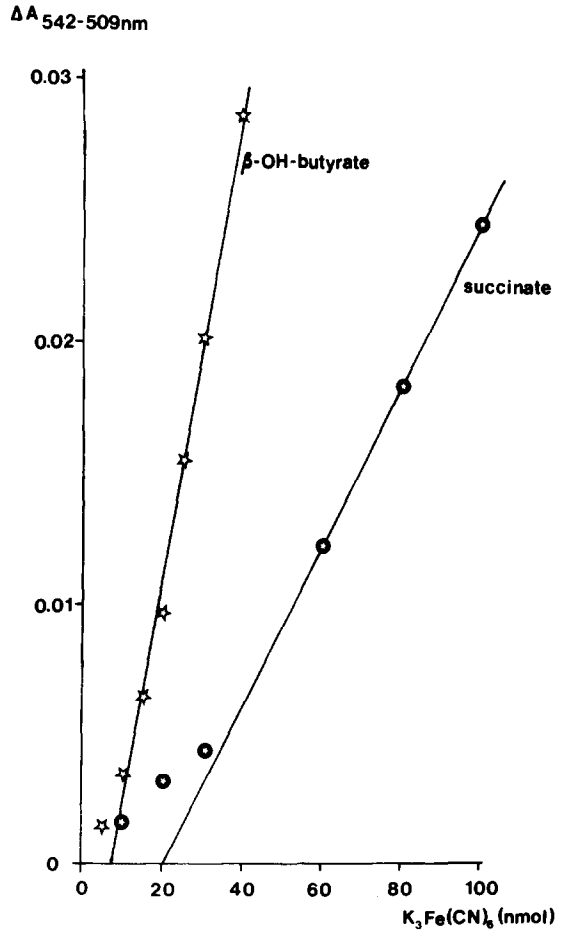

Fig.3. Extent of charge displacement with succinate and β-OH-butyrate as substrate. The conditions were those described in the legend to fig.2, except that 38 μM safranine replaced the neutral red and the valinomycin was omitted. The extent of absorption change is plotted *vs* the amount of added potassium ferricyanide.

efficiency of site 1 is twice that proposed originally in [13], and is inconsistent with the directly coupled redox loop model of the NADH-ubiquinone reductase segment suggested by the latter authors.

Although it seems very likely on the basis of the control experiments and the earlier data of others (see above) that NR and safranine measure changes in matrix pH and electrical charge displacement, respectively, the interpretation is, in fact, independent of these notions. Since the absorption changes of both NR and safranine are fully energy-dependent, the results show in any case that the energy-conserving efficiency of site 1 is twice that of site 2. Thus, if it is accepted that energy conservation occurs by proton translocation, the H^+/e^- stoichiometry for oxidation of NADH by ubiquinone is twice as high as for oxidation of succinate by ferricytochrome *c*.

Whether NR measures matrix pH or changes in H^+ activity more localised to the membrane is a relevant question (see [5,6,17]). Although most of the NR may be bound to the membrane it has nevertheless been concluded that it reflects the delocalised intravesicular pH [5,6,17], at least on the seconds time scale relevant here, due to fast proton equilibration between the matrix and the regions of the membrane occupied by the dye. On the basis of the present data there is no reason to assume that this would not be the case. The partial 'quenching' of the NR signal in the absence of NEM (allowing transport of P_i in symport with H^+), and the further decrease on adding extraneous P_i, support the conclusion that the delocalised matrix pH is measured in the conditions employed.

4. GENERAL CONSEQUENCES

The respiratory chain segments between NADH and Q, and cytochrome *c* and O_2 are twice as efficient in energy conservation per transferred electron than the segment between QH_2 and cytochrome *c* (here and [9]). Authors in [18] recently presented convincing evidence that the respiratory chain between NADH and cytochrome *c* is very close to thermodynamic equilibrium with the phosphorylation system in State 4 mitochondria, and that the $ATP/2e^-$ ratio is 2.0 for this segment (i.e., sites 1 + 2). If the chemiosmotic notion of coupling respiration to phosphorylation through proton circuits is accepted, this means that the H^+/ATP ratio for the phosphorylation of extramitochondrial ADP to ATP equals the effective H^+/e^- ratio for this segment of the chain. According to the data presented here the former ratio is then 3 H^+/ATP. It may therefore be predicted that the maximal $ATP/2e^-$ ratios for sites 1, 2 and 3 are 4/3, 2/3 and 4/3, respectively.

5. CONCLUSIONS

The present method is the first quantitative determination of proton uptake from the mitochondrial matrix during proton translocation. The results

show that two protons are taken up from the matrix, and that two electrical charge equivalents are translocated across the membrane per electron transferred between NADH and ubiquinone (site 1). This is in agreement with and complements the proposals in [15,16] for site 1, which were based on measurements of proton release from mitochondria.

These results, in conjunction with those in [9,18] may be used to estimate the H^+/ATP ratio of ATP synthesis and the maximal $ATP/2e^-$ stoichiometries for the different sites of the respiratory chain.

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